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Interleukin-1α downregulates extracellular-superoxide dismutase in human corneal keratoconus stromal cells

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Purpose: The purpose of this investigation was to elucidate the regulation of corneal extracellular superoxide dismutase (SOD3) synthesis in keratoconus. We compared the basal and cytokine-regulated SOD3 synthesis in cultured human stromal cells from keratoconus corneas to stromal cells from normal and bullous keratopathy corneas.
Methods: Keratocyte cultures were obtained from patients undergoing corneal transplantation for keratoconus and bullous keratopathy, and from healthy donor corneas. The cell lines obtained were cultured until near confluence and interleukin-1α, interleukin-6, transforming growth factor β, or platelet derived growth factor were added to the media. The phenotypes of the cultured cells were assessed by immunocytochemical expression of α-smooth muscle actin and CD34. SOD3 protein contents were determined in the culture media with ELISA after 24, 48, 72, and 96 h.
Results: Interleukin-1α had an inhibitory effect on SOD3 synthesis exclusively in the keratoconus cultures ($p<0.01$). Platelet derived growth factor induced a reduction in SOD3 synthesis in all groups ($p<0.05$).
Conclusions: Here, we demonstrate that cultured keratoconus stromal cells respond with a reduced SOD3 synthesis to interleukin-1α, which is not the case in corresponding normal or bullous keratopathy cells. Since interleukin-1α is upregulated in corneal trauma and inflammation, keratoconus corneas may muster an insufficient oxidative defense under such conditions.

The stroma of the human cornea has a slow tissue turnover rate and absorbs a large part of the ultraviolet light entering the eye. Also, the high demands of the eye's optical system call for a very exact tissue organization in the corneal stroma. As a consequence, the cornea is potentially vulnerable to oxidative stress. The cytosolic copper-zinc superoxide dismutase (SOD1) [1] and the extracellular-superoxide dismutase (SOD3) [2] are approximately equally abundant in the human cornea [3]. The substrate of the SOD isoenzymes, the superoxide anion, penetrates membranes poorly. The SOD isoenzymes therefore exert their actions in their respective compartments - SOD1 within the cells and SOD3 in the extracellular space. Since the extracellular space comprises a large proportion of the cornea and in particular the corneal stroma [4], SOD3 is likely to be the major superoxide scavenger in this tissue [3].

Oxidative stress is thought to contribute to the pathogenesis of the non-inflammatory ectatic corneal degeneration keratoconus (KC) [5-9]. Although the SOD3 mRNA levels in keratoconus are unaltered [8], the corneal levels of SOD3 enzyme are halved [10], which is likely to increase the risk of oxidative damage in the corneal stroma in KC.

In the present study, our aim was to elucidate the mechanisms regulating corneal stromal SOD3 synthesis in KC utilizing a model based on cultured stromal cells from patients with KC. As controls, we used stromal cells from normal corneas and from corneas with bullous keratopathy (BKP). BKP is a commonly occurring corneal disease with corneal edema caused by insufficient corneal endothelial pump function [11]. We have previously shown that the overall SOD3 levels in the cornea are unaltered in BKP after correction for the tissue edema [10]. We chose to use corneas from another corneal disease as an additional control in the present investigation since these corneas can be handled exactly like the KC corneas, while some post mortem time is inevitable with the normal control corneas. In addition, deviations in the SOD3 synthesis response occurring in the KC cultures, but not in the BKP or normal controls, are more likely to be specific for KC rather than an unspecific finding seen in any corneal disease.

The SOD3 synthesis in dermal fibroblasts and in smooth muscle cells is not regulated directly by oxidative stress but rather via inflammatory cytokines and growth factors [12-14]. Therefore, we chose to quantify the SOD3 synthesis in the presence of various cytokines and growth factors. The cytokines and growth factors investigated here have been shown earlier to regulate SOD3 synthesis in other cell types [12-14] and also have a putative relevance to corneal disease processes [15-18].

METHODS

Corneas: The tenets of the Declaration of Helsinki for the collection of human material were followed in this investigation and the research ethics committee of Umeå University, Umeå , Sweden approved the study. Since our previous investigations on skin fibroblasts and smooth muscle cells have dem-

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onstrated a large variability in the SOD3 synthesis between cell lines [12,13], we included multiple cell lines in each group. KC (n=9) and BKP (n=7) keratocytes were obtained from patients undergoing penetrating keratoplasty. No signs of active inflammation were seen in any of these eyes and none of the KC cases were secondary to an underlying connective tissue disease or associated with Down's syndrome. In the BKP group, two eyes were phakic with Fuchs' dystrophy as the primary cause of the edema and five were pseudophakic. Normal human corneal stromal cell cultures were obtained from donor corneas where the donor had approved the donation for research purposes (n=8). In one case, the cells were obtained from a cornea transplant because of an inactive, central traumatic scar.

Cell cultures: Small pieces of corneal stroma were cut from the corneal mid-periphery (3.5-4.0 mm from the center) and placed in cell culture flasks in 50:50 Dulbecco's MEM: Hams F12 (Gibco, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 72 μg benzylpenicillin/ml. The culture medium was changed twice every week and the cells were grown to near confluence. The cells were then either kept at -180 °C until the investigations or immediately seeded into 12-well culture plates with a bottom area of 3.80 cm^2. The cells were again grown until near confluence. The experiments were performed under low stimulation conditions with the FCS concentration reduced to 0.5%. The culture media contained the cytokines listed below or no cytokines according to our previously used protocol [12,13]. During the experiment, the medium was replaced daily with a fresh medium containing cytokines. The collected medium was kept at -80 °C until analysis (see below). After 96 h, the plates were washed three times in 0.15 M NaCl, then 0.5 ml ice-cold 50 mM Na phosphate (pH 7.4) with 0.3 mM KBr, 10 mM diethylene-triamine pentaacetic acid, 0.5 mM phenylmethylsulfonyl fluoride, and 100 KIU/ml aprotinin (the latter three for protease inhibition) were added to each well. After sonication with the plates kept on ice and centrifugation of the cell homogenates at 20.000x g for 10 min, the supernatants were collected for analyses. Collected media and cell supernatants were kept at -80 °C until analysis. The experiments were performed in duplicates and the means of the two twin cultures were used in the calculations.

Cytokines: Recombinant human interleukin-1α (IL-1α) was obtained from Genzyme Corp. Boston, MA, USA; interleukin-6 (IL-6) from R&D, Minneapolis, MN, USA, and transforming growth factor β (TGF-β), basic fibroblast growth factor (b-FGF), and platelet derived growth factor (PDGF) were obtained from Sigma, St Louis, MO, USA. The cell cultures were supplemented with 50 U/ml IL-1α, 2 ng/ml IL-6, 0.30 ng/ml TGF-β, 50 ng/ml b-FGF or 100 ng/ml PDGF. Wells without cytokines were used as controls.

Immunocytochemistry: To determine the phenotype of the cultured cells, two cell lines each from KC, BKP, and normal corneas were cultured under the conditions described above on chamber covered glass slides. These cultures were subject to one of four treatments: 50 U/ml IL-1α, 100 ng/ml PDGF, no cytokines + 0.5% FCS, or no cytokines + 10% FCS. The media of these cultures were also collected daily during the 96 h and kept for SOD3 analysis (see below). After 96 h, the cells were washed in PBS 3x5 min, fixed in 2% paraformaldehyde for five min, washed again in PBS 3x5 min, and kept in PBS until processed for immunocytochemistry with either mouse monoclonal antibodies against human CD34 (Novocastra, Newcastle upon Tyne, UK) diluted 1:10 or mouse monoclonal antibodies against alpha-smooth muscle actin (α-SMA; Dako, Glostrup, Denmark) diluted 1:50. The cells were incubated with the primary antibody for one h at 37 °C. Control cultures were treated as above but without the primary antibody.

Analyses: The SOD3 protein contents were determined with an ELISA as previously detailed [3]. In all calculations, the SOD3 contents were related to the protein and DNA contents of the cultures obtained at 96 h. For protein analysis, Coomassie Brilliant Blue G-250 was employed, standardized with human serum albumin [19]. The DNA contents were determined with fluorimetry as a complex with bisbenzimidazole (Hoechst 33258) [20] using calf thymus DNA as a standard.

The Cytotox 96® kit (Promega Corp., Madison, WI) was used as an indicator of unspecific cell damage by determining the leakage of lactate dehydrogenase (LDH) from the cultured cells at 96 h. The ratio of the LDH activity in the medium to the LDH activity in the cell supernatant was calculated for each culture.

Student's t-tests for paired and unpaired values were used for longitudinal and between group comparisons, respectively. Appropriate Bonferroni corrections were performed for the comparisons between groups. For correlations, Spearman's rho was used. A level of $p<0.05$ was considered statistically significant.

RESULTS

Immunocytochemistry: The vast majority of the cells were unlabeled with the antibodies against α-SMA. Sporadic cells in all cultures displayed a moderate to strong staining (Figure 1). The very few cells expressing α-SMA were likely too few to contribute to the overall SOD3 synthesis of the cultures. Cells with detectable staining for CD34 were even fewer (Figure 1). This pattern of staining did not differ between the KC, BKP, and normal cultures, between the cytokines, or between 0.5% and 10% FCS (Figure 1).

SOD3 synthesis: To correct for differences in cell density between wells, all SOD3 data were correlated to cell protein or DNA determined at 96 h. An approximate doubling of the basal SOD3 synthesis was seen from 24 h (21.6±14.7 μg/g protein/24 h, means±SD) to 96 h (42.0±24.0 μg/g protein/24 h, means±SD) for the whole material. We found no significant differences in basal SOD3 synthesis between the three groups in the present investigation.

Interestingly, IL-1α had an inhibitory effect on the SOD3 synthesis exclusively in the KC cells (43±6% of the corresponding untreated KC control wells at 96 h, means±SD, range 10-61%; $p<0.001$; Table 1, Figure 2). In the normal cells, the SOD3 synthesis ranged from 64-170% of untreated control wells ($p=0.15$) and in the BKP cells, the SOD3 synthesis ranged

from 77-264% of the untreated control wells (p=0.12). Both in normal and BKP cells, there was a significant increase in SOD3 synthesis over the course of the experiment with IL-1αcompared to control wells but not in the KC group (Figure 2). In all groups, IL-1α induced a slight increase in protein contents (121±28% for the whole material; p<0.001) but no change in cell proliferation as assessed with DNA content was seen.

PDGF induced a substantial decrease in SOD3 synthesis, similar in all three groups (20±11% of untreated control wells, means±SD, at 96 h for the whole material; Table 1). A small increase in cell proliferation as assessed with DNA contents (120±18% of untreated control wells, means±SD; p<0.05) and a more pronounced increase in protein contents (194±52%; p<0.001) were also seen in all groups with PDGF. In the whole material, a strong correlation was found between the cellular

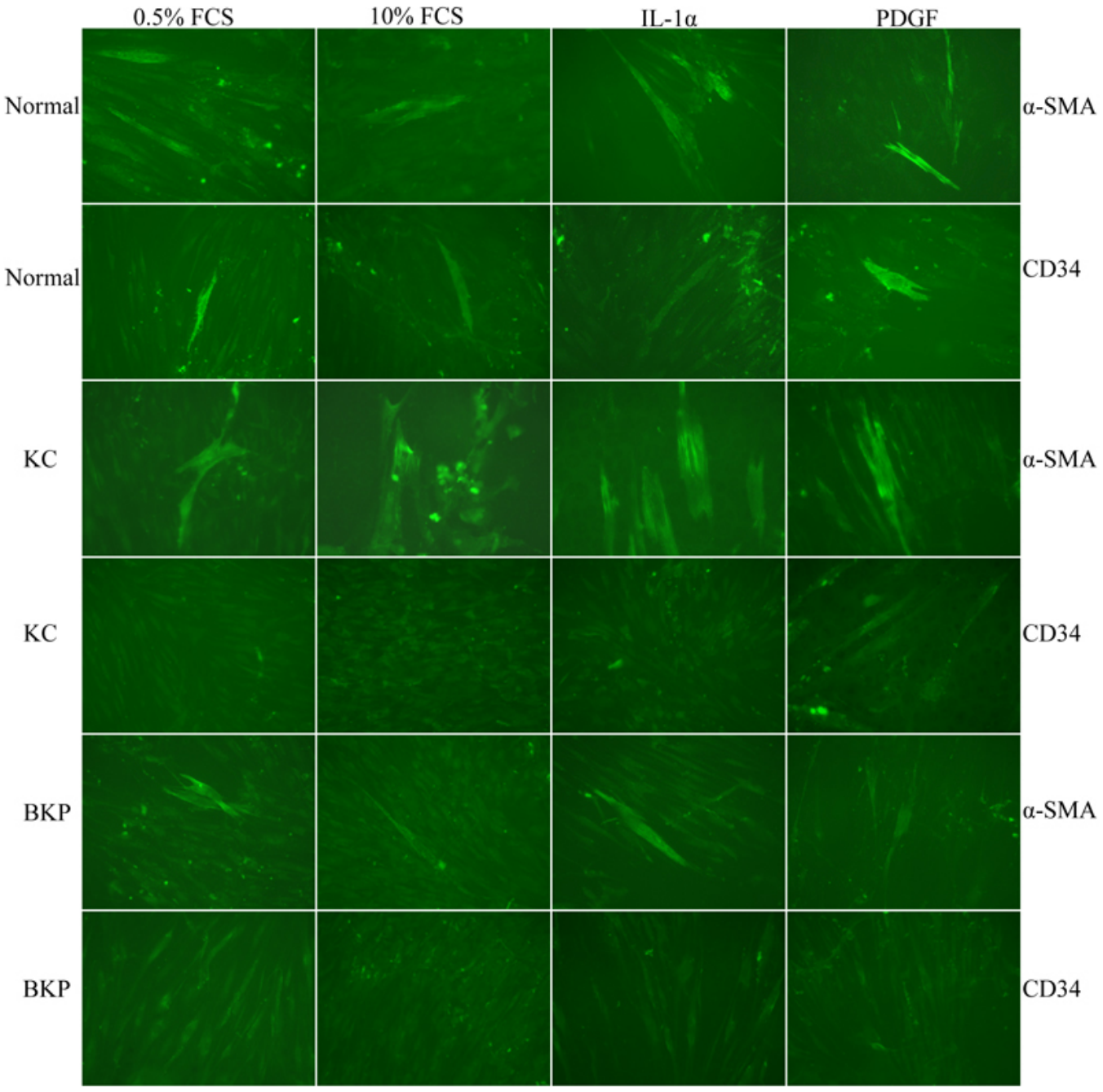

Figure 1. Cultured corneal stromal cells. Cultured stromal cells from a normal cornea, a cornea with keratoconus, and a cornea with bullous keratopathy were all stained with mouse monoclonal antibodies against α-SMA and CD34 after 96 h of culture in 0.5% or 10% FCS. The vast majority of the cells were unlabeled by the two antibodies. A few cells were labeled with the α-SMA antibody and even fewer with the CD34 antibody. No differences were seen between groups or treatments. The staining pattern suggests that the cells were of a fibroblast phenotype.

SOD3 contents and the SOD3 contents in the media at 96 h (correlation coefficient=0.854; p<0.001).

Smaller yet significant changes in SOD3 synthesis were also seen with TGF-β and IL-6 (Table 1). TGF-β reduced the SOD3 synthesis both in normal cultures (57±16% of untreated control wells, means±SD, at 96 h; p=0.018) and KC cultures (52±9%; p=0.004) but not in BKP cultures. IL-6 increased the SOD3 synthesis slightly in BKP cultures (154±22% of untreated control wells, means±SD, at 96 h; p=0.014) and decreased the SOD3 synthesis slightly in KC cells (87±10%; p=0.025) but had no significant effect on normal cells. B-FGF did not affect the SOD3 synthesis. The SOD3 synthesis pattern in the cultures used for immunocytochemistry, as well as the effects of the cytokines, were as described above (data not shown).

Cytotoxicity: No signs of unspecific cell damage were seen in any group or with any cytokine treatment. In the whole material, the leakage of LDH from 72 h to 96 h ranged between 0 and 4.6% of the corresponding cellular LDH contents.

DISCUSSION

We demonstrated that IL-1α downregulates the synthesis of SOD3 in cultured KC stromal cells but not in the corresponding normal or bullous keratopathy cells. The immunocytological staining pattern of the cells suggests a fibroblast phenotype regardless of treatment, resembling the situation in a corneal wound healing process. The finding of a downregulation of SOD3 synthesis by IL-1α exclusively in the KC stromal cells is of particular interest since IL-1α has

Table 1. Levels of SOD3 in the culture media and cell layers in bullous keratopathy, keratoconus and normal fibroblastic keratocyte cell lines

	SOD3, μg/g protein					SOD3, mg/g DNA				
	24 h	48 h	72 h	96 h	Cells	24 h	48 h	72 h	96 h	Cells
Untreated controls										
Bullous keratopathy	27.5±5.6	38.1±10.3	44.0±9.9	48.1±10.3	12.2±2.4	0.24±0.06	0.32±0.08	0.36±0.07	0.39±0.07	0.07±0.02
Keratoconus	20.6±4.5	26.1±4.2	38.3±4.8	50.0±5.7	5.6±1.2	0.24±0.09	0.29±0.09	0.41±0.10	0.53±0.12	0.16±0.06
Normal	19.2±6.2	24.3±7.6	27.6±8.7	31.5±8.8	5.6±1.2	0.17±0.06	0.23±0.08	0.26±0.09	0.28±0.09	0.05±0.01
PDGF, 100 ng/ml										
Bullous keratopathy	11.8±2.7*	13.0±3.4*	12.6±3.0*	11.8±3.1*	4.3±1.0*	0.16±0.04*	0.17±0.05*	0.16±0.04*	0.15±0.04*	0.05±0.01*
Keratoconus	8.7±2.0*	8.5±2.0*	8.2±1.8*	9.6±2.3*	2.3±0.3*	0.12±0.03*	0.11±0.03*	0.11±0.03*	0.12±0.04*	0.06±0.02*
Normal	5.7±1.6*	5.7±1.5*	5.7±1.5*	5.4±1.4*	2.3±0.3*	0.09±0.03*	0.09±0.03*	0.09±0.02	0.08±0.02*	0.03±0.01
IL-1α, 50U/ml										
Bullous keratopathy	20.1±3.6*	36.6±5.5	51.9 ±6.7	64.0±7.2	7.3±1.9*	0.20±0.04	0.36±0.06	0.50±0.05*	0.62±0.05*	0.15±0.02*
Keratoconus	20.3±7.2	18.2±3.4*	18.2±4.0*#	22.2±4.4*#	8.7±2.1	0.14±0.03*	0.16±0.04*	0.19±0.04*#	0.23±0.05*#	0.08±0.02
Normal	11.8±3.5*	18.7±6.0*	30.8±9.9	36.5±10.4	8.7±2.1	0.12±0.03	0.19±0.06	0.32±0.10*	0.38±0.11*	0.09±0.03
TGF-β, 50 ng/ml										
Bullous keratopathy	24.3±4.5	32.6±8.8	39.2±9.6	45.6±11.0	7.1±1.2	0.22±0.06	0.30±0.10	0.35±0.10	0.41±0.12	0.07±0.01
Keratoconus	19.4±6.2	15.9±3.5*	22.3±3.7*	30.5±5.2*	4.1±1.1	0.15±0.04	0.16±0.04*	0.23±0.05*	0.30±0.07*	0.07±0.02*
Normal	14.5±4.9	18.9±6.3	19.5±7.5*	21.2±8.9*	4.1±1.1	0.13±0.05	0.18±0.06	0.19±0.08	0.21±0.09*	0.04±0.01*
IL-6, 0.30 ng/ml										
Bullous keratopathy	33.7±6.6*	50.6±11.5*	63.8±11.3*	71.9±14.5*	8.8±1.8	0.20±0.04	0.36±0.06	0.50±0.05	0.62±0.05	0.15±0.02
Keratoconus	31.8±14.4	23.4±4.9	34.8±4.4	39.2±5.9*	5.0±1.1	0.14±0.03	0.16±0.04	0.19±0.04	0.23±0.05*	0.08±0.02*
Normal	22.5±7.4	26.5±8.4	27.2±8.5	26.8±8.2		0.12±0.03	0.19±0.06	0.32±0.10	0.38±0.11	0.09±0.03*

The levels of SOD3 in the culture media and cell layers in bullous keratopathy (n=7), keratoconus (n=9), and normal (n=8) fibroblastic keratocyte cell lines after 24-96 h of culture, means ±SEM. All SOD3 levels are normalized to the protein or DNA levels present in the cell layer after 96 h of culture. The asterisk indicates significant change compared to the corresponding untreated control wells (p<0.05); the hash mark indicates that the induced change from the corresponding, untreated control wells differs significantly from both other groups (p<0.05).

been proposed as a master regulator of the corneal wound healing response [21]. IL-1α, being constitutively expressed by the corneal epithelium [21], is dramatically upregulated via an autocrine loop [16] in the early phase of the corneal wound healing cascade [22]. The KC cornea shows signs of augmented oxidative stress with increased formation of both nitrotyrosine and malondialdehyde [6] particularly in areas with fibrosis where a healing process takes place [6]. In recent years, improved KC diagnostics [23] and new knowledge in the field of KC genetics [24-26] indicate that the genetic factors underlying the disease are unquestionable but complex. Based on the present findings, one can hypothesize that the release of the master wound healing regulator, IL-1α, in the cornea by any cause in an individual genetically predisposed for KC may cause a paradoxical reduction of the SOD3 synthesis in the corneal stroma. A reduced SOD3 synthesis may in turn contribute to an augmented oxidative stress, which has been proposed to result in a cascade of events involving cell apoptosis, collagen resorption, tissue degradation, and corneal thinning according to the cascade hypothesis of KC pathogenesis [5-8,17].

In the present study, the basal SOD3 synthesis in cultured corneal stromal cells was in the same range as previously reported for cultured human dermal fibroblasts [12] and smooth muscle cells [13]. In these two cell types, the SOD3 synthesis is not regulated directly by its substrate or other forms of oxidative stress but rather via cytokines and growth factors [12]. The overall cytokine-mediated effects on SOD3 synthesis in corneal stromal cells showed a similar, slow development over several days as previously reported for dermal fibroblasts and smooth muscle cells [12,13] but with different effects for individual cytokines. For example, of the cytokines evaluated in the present study, IL-1? had only small, variable effects on the SOD3 synthesis in smooth muscle cells while TGF-β and IL-6 did not alter the SOD3 synthesis significantly. The increase in basal SOD3 synthesis seen over the course of the present experiment is also similar to our previous findings in skin fibroblasts and smooth muscle cells [12,13]. The large variations seen in the basal- and cytokine-stimulated SOD3 synthesis between different cell lines is also in accordance with our previous findings in other cell types [12,13]. The strong correlation seen between the cellular SOD3 contents and the SOD3 contents in the culture media demonstrates against "secretion blocking" being a major mechanism underlying the reduced extracellular SOD3 levels seen with, for example, PDGF.

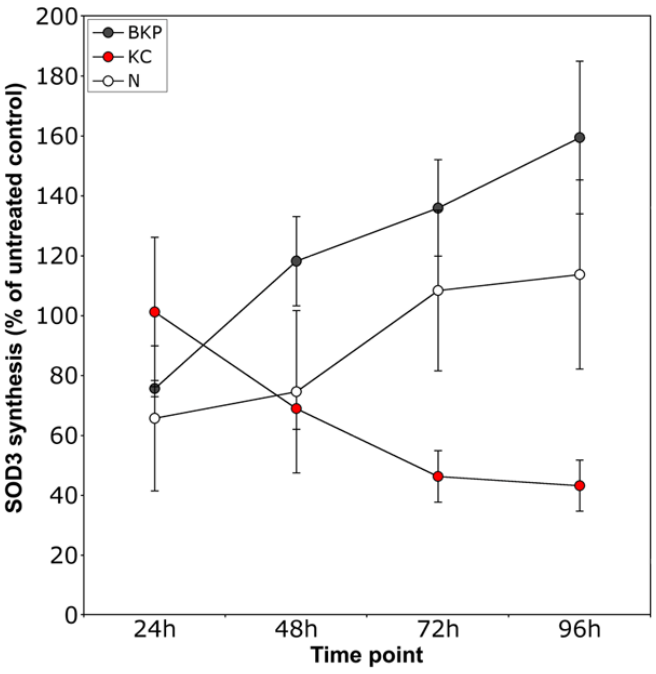

Figure 2. SOD3 synthesis in bullous keratopathy, keratoconus, and normal cultured stromal cells. SOD3 synthesis in bullous keratopathy (BKP), keratoconus (KC), and normal (N) cultured stromal cells after 24-96 h of culture with 50 U/ml IL-1α, expressed as a percent of untreated control wells (Means±SEM). The SOD3 synthesis in keratoconus cells is significantly lower than that of bullous keratopathy cells from 48 h ($p=0.047$) and significantly lower than both of the other groups from 72 h ($p<0.01$).

The present investigation was performed with early-passage cultures of cells (two to four passages) under low serum conditions rather than with totally unstimulated primary keratocyte cultures. Some differences have been demonstrated between early-passage and primary keratocyte cultures [16]. Keratocytes, cultured under low serum conditions, such as in the present study are sometimes assumed to exhibit a keratocyte rather than a fibroblastic or myofibroblastic phenotype [21] but the general lack of both α-SMA and CD34 expression suggests a fibroblastic phenotype of the cells in the present investigation. Still, the increase in SOD3 synthesis, demonstrated over the course of the experiment, could reflect a return to a more "quiescent" cell phenotype although a few cells show expression of α-SMA at 96 h. Extrapolation of these data suggests that the basal synthesis of SOD3 in the corneal stroma may be rather high, which is in line with our previous findings of unusually high SOD3 levels in the cornea [3,10].

We also show that PDGF, known to initiate corneal fibroblast chemotaxis and proliferation, invariably downregulates the SOD3 synthesis compared to the untreated control cultures. The SOD3 synthesis in the PDGF-treated fibroblasts was unaltered over the course of the experiment while the synthesis increased in the untreated control cultures. The effect of PDGF might thus reflect a preservation of the SOD3 synthesis levels seen with serum-enriched media.

The present study's findings of an abnormal downregulation in the SOD3 synthesis in cultured KC fibroblasts by IL-1α bring further support to previous studies indicating an abnormal processing of the superoxide radical in the keratoconus cornea and an involvement of oxidative stress in the pathogenesis of this disease.

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