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ORIGINAL PAPER

Elevated lipocalin-2 level in aqueous humor of patients with central retinal vein occlusion

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Abstract
Purpose To assess the concentrations of lipocalin-2 (LCN2) in the serum and the aqueous humor of patients with central retinal vein occlusion (CRVO).
Methods The concentrations of LCN2 in the serum and aqueous humor of 16 cataract patients and 16 patients with CRVO with macular edema were compared. Collection of aqueous samples was conducted in the operating theater under sterile conditions and just prior to intravitreal ranibizumab injection or cataract surgery. LCN2 levels in serum and aqueous humor samples were measured using a commercial kit (human lipocalin-2/NGAL PicoKine ELISA Kit, MyBioSource Inc., USA; Catalog No: MBS175829) based on standard sandwich enzyme-linked immunosorbent assay technology.
Results The concentrations of LCN2 in the aqueous humors of the CRVO group were higher than those of the control group ($p = 0.021$). There was no significant difference in serum LCN2 level between the two groups ($p = 0.463$).
Conclusions Concentrations of LCN2 in aqueous humor are increased in CRVO. LCN2 may be part of a pro-catabolic phenotype, and it may play an important role in the dreaded complications of CRVO, such as macular edema, macular ischemia, and neovascularization, which lead to blindness.

Keywords Aqueous humor · Lipocalin-2 · Macular edema · Matrix metalloproteinase · Retinal vein occlusion

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Introduction

Retinal vein occlusion (RVO) is the second most common cause of retinal vascular disease after diabetic retinopathy and is a common cause of significant visual reduction and late ocular complications [1]. Although intraluminal thrombus formation secondary to several conditions such as diabetes, hyperlipidemia, hypertension, and thrombophilia is the most important event, the pathogenesis of RVO is not yet fully understood [2, 3]. A number of recent studies show that inflammation may play a role in the molecular pathways responsible for the vision-impairing consequences of RVO, such as retinal ischemia and macular edema, the severity of which has been found to be associated with various inflammatory cytokines in vitreous or aqueous humor samples of patients with RVO [4, 5].

A number of cytokines including vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) have the strongest evidence of association with central retinal vein occlusion (CRVO) [5]. However, their concentrations often do not correspond to the associated clinical CRVO phenotype. Although VEGF is a target to reduce macular edema in patients with CRVO, it is argued that VEGF inhibition alone may not be sufficient to decrease the inflammatory response in CRVO therapy [6].

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL), is an adipose-derived cytokine and is involved in a series of processes such as modulation of inflammation and metabolic homeostasis [7]. It was suggested that LCN2 acts in an autocrine manner to induce cell death sensitization and morphological changes in cells under inflammatory conditions and that these phenotypic changes may be the basis of reactive changes in vivo [8]. The aim of this study was to evaluate the levels of LCN2 in serum and aqueous humor of patients with central retinal vein occlusion (CRVO).

Materials and methods

Sixteen patients (11 males, 5 females) with CRVO aged 56–75 years and 16 healthy control subjects (11 males, 5 females) aged 66–83 years were enrolled in this study. The study included non-diabetic, treatment-naive patients with CRVO with macular edema. The control group consisted of non-diabetic cataract patients requiring cataract surgery. Patients were enrolled in the study after undergoing systemic evaluation and ocular examination. All the patients were carefully examined with slit lamp before surgery, and detailed fundus examinations were made by using a contact fundus lens. Color fundus photographs, fluorescein angiograms (FFA), and macular scans with time-domain optical coherence tomography (OCT) were taken routinely 1 week before intravitreal ranibizumab (IVR) injection or surgery. Retinal ischemia was evaluated by measuring the area of capillary non-perfusion using FFA, and macular edema was examined by OCT.

Exclusion criteria: (1) uncontrolled hypertension (systolic and diastolic blood pressure greater than 160 or 100 mmHg, respectively); (2) diabetes mellitus; (3) previous intraocular surgery or laser photocoagulation within the past 6 months; (4) associated uveal or retinal pathology other than CRVO; (5) glaucomatous eyes; (6) eyes with ‘rubeosis iridis’; (7) vitrectomized eyes; and (8) previous intravitreal injection of steroid or an anti-VEGF agent. Written informed consent was obtained from each patient at least 24 h prior to surgery. The study was performed following a protocol that was approved by our hospital’s institutional ethics committee (27.04.2016/46), conforming to the ethical principles of the Declaration of Helsinki.

Undiluted aqueous humor samples were collected from 16 eyes of 16 individuals undergoing intravitreal injection of ranibizumab for the treatment of symptomatic and active macular edema and requiring anterior chamber paracentesis. Aqueous humor samples were aspirated to prevent a surge in intraocular pressure after the intravitreal injection of ranibizumab. They were naive cases and had not been treated in the past. For the controls, aqueous humor samples from 16 eyes of 16 patients with senile cataract who underwent cataract surgery were obtained. The control group was age-matched with the CRVO group.

Collection of aqueous samples was conducted in the operating theater under sterile conditions and just prior to intravitreal ranibizumab (IVR) injection or cataract surgery. Samples (0.1–0.2 mL) of aqueous humor were collected in sterile tubes by way of limbal anterior chamber puncture with a 27-gauge needle from a 1-ml insulin injector. The specimens were transferred to sterile tubes and stored at − 20 °C until assayed.

After clotting, blood samples were centrifuged at 1000 g for 15 min, and the serum supernatant removed and aliquoted, and all serum and aqueous humor samples were stored at − 20 °C until assayed. LCN2 levels in serum and aqueous humor samples were measured using a commercial kit (human lipocalin-2/NGAL, PicoKine ELISA Kit, MyBioSource Inc., USA; Catalog No:MBS175829) based on standard sandwich enzyme-linked immunosorbent assay technology.

Statistical analysis

SPSS 11.5 (SPSS Inc, USA) software was used to perform the statistical analyses. Ages and LCN2 levels were compared with Mann–Whitney *U* test. Fisher’s exact test was used to compare the gender distribution

between patients and controls. The differences were considered significant at $p < 0.05$.

Results

A total of 32 aqueous humor samples from 16 diseased patients and 16 controls were collected. No statistically significant differences were found among the mean ages in the CRVO (mean ± SD; 63.3 ± 8.4 years, range 56–75 years) and cataract control (74.3 ± 6.2, range 66–83) groups ($p = 0.065$). Gender distribution was similar between the patient and control groups ($p = 1.000$). The mean duration of CRVO was 3.8 ± 2.8 months.

The age and gender distribution are shown in Table 1.

The aqueous humor level of LCN2 was 59,053 ± 39994 (mean ± SD) pg/ml in eyes of patients with CRVO and 1652 ± 64 pg/ml in eyes of the control group. The mean aqueous humor LCN2 level in CRVO patients was significantly higher than that of the controls ($p = 0.021$). The serum level of LCN2 was 96,035 ± 10,883 (mean ± SD) pg/ml in patients with CRVO and 91,838 ± 12,228 pg/ml in the control group. The mean LCN2 level in the serum of CRVO patients was also higher than healthy subjects, but the difference was not significant ($p = 0.463$) (Table 2).

Discussion

In an attempt to contribute to the understanding of the role that LCN2 may exert in CRVO, in this study we quantified serum and aqueous humor LCN2 concentrations in both healthy subjects and patients with CRVO. Our results showed a significant elevation of aqueous humor LCN2 levels in patients with CRVO in comparison with the levels observed in the aqueous humor of healthy subjects. However, there was no significant difference between the two groups in terms of the serum LCN2 levels (Table 2). To the best of our knowledge, the present study is the first to investigate LCN2 levels in patients with CRVO.

LCN2 is an acute-phase protein and was originally identified in human neutrophil granules. In addition to neutrophils, it is expressed in several other tissues, including adipocytes, macrophages, liver, lung, kidney, and retina pigment epithelium (RPE) cells [9–12]. As an acute-phase protein, LCN2 has become increasingly relevant as a potential biomarker for inflammatory diseases [7]. LCN2 levels have been shown to increase in both serum and tissues during inflammation [13]. Marques et al. suggested that LCN2 was produced by the choroid plexus as a component of the innate immune response and increased with infections of the central nervous system (CNS) [14]. Increased expression and the important role of LCN2 in various pathological states, including cancerous conditions, kidney diseases, cardiovascular diseases, and diabetes, have been shown [15–18].

For the first time, the elevated concentration of LCN2 in aqueous humor was shown in patients with idiopathic acute anterior uveitis and these results implied that LCN2 was associated with the regulation of ocular inflammation [19]. Previous studies identified *LCN2* as a gene in the retinal ganglion cell layer significantly and consistently upregulated in an early glaucoma model injury [20, 21]. Valapala et al. and Parmar et al. showed that *LCN2* was the most upregulated early stress response gene identified in a mouse model with age-related macular degeneration-like pathology [11, 12]. Protein LCN2 was produced by RPE cells and the neural retina after intense light exposure as well as in cultured RPE cells from mice and humans incubated with lipopolysaccharide or photoreceptor outer segments [12].

Table 1 Baseline characteristics of groups

Parameters	CRVO group	Control group	*p*
Age (years)	63.3 ± 8.4 years	74.3 ± 6.2	1.000
Sex ratio (male/female)	11/5	11/5	0.065
CMT (μm)	374.9 ± 91.2	212.5 ± 16.1	< 0.001

CRVO central retinal vein occlusion, *CMT* central macular thickness

Table 2 Serum and aqueous humor LCN2 levels of the patients and the controls

	Serum LCN2 levels (pg/ml)		Aqueous humor LCN2 levels (pg/ml)	
	Mean ± SD	Median (min–max)	Mean ± SD	Median (min–max)
CRVO group	96,035 ± 10,883	100,279 (80,596–104,920)	59,053 ± 39,994	29,394 (1710–95,713)
Control group	91,838 ± 12,228	94,303 (68,674–104,920)	1652 ± 64	1675 (1560–1699)
p	0.463		0.021*	

LCN2 lipocalin-2, *Min* minimum, *Max* maximum, *SD* standard deviation

*A significant difference

Hangai et al. showed that *IL-1β* gene was expressed by retinal glial cells, endothelial cells, and neutrophils in the retina after transient ischemia [22]. Zhao et al. reported that the concentrations of IL-1β in vitreous samples of patients with RVO were significantly elevated when compared with a control group [23]. It is known that expression of LCN2 is strongly upregulated by *IL-1β* and increased levels of IL-1β in RVO may be one of the reasons for the high LCN2 levels found in our patients [24–26]. LCN2 increase in CRVO patients was the first result observed and will form a firm foundation for more advanced and comprehensive studies encompassing IL-1β.

The function of LCN2 in the eye and ocular tissues is currently unknown. However, its activity is most probably related to one or more of the multifaceted functions of this molecule identified in other tissues. After inducing temporary ischemia in mice, Jim et al. investigated brain damage and reported that after ischemia LCN2 increased especially in endothelial cells and astrocytes. When they compared mice with LCN2 insufficiency with healthy mice, the volume of brain infarctus, release of inflammatory mediators, and blood–brain barrier permeability were significantly lower in animals with LCN2 insufficiency [27]. Retinal iron accumulation leads to abnormal retinal angiogenesis/vasculogenesis, with proliferation of new, leaky blood vessels in the vitreous [28]. Yang et al. showed that LCN2 increased the entry of iron into cell cytoplasm [29].

LCN2 protects matrix metalloproteinase (MMP)-9 (gelatinase B) from proteolytic degradation and in this way enhances its enzymatic activity by forming the MMP-9/LCN2 complex [30]. MMP-9 directly degrades extracellular matrix (ECM) proteins and activates cytokines and chemokines to regulate tissue remodeling in multiple physiological and pathological processes such as angiogenesis, inflammation, and fibrosis [31]. In a recent study, it was shown that, compared to controls, the concentration of MMP-9 was increased in the aqueous humor of diabetic macular edema (DME) patients suggesting that MMP-9 may be involved in the pathogenesis of DME and this change was prominently found in patients with proliferative diabetic retinopathy and in proportion to the duration of diabetes mellitus [32]. Tuuminen et al. found that MMP-9 levels increased in three CRVO and one branch RVO eyes. It has been hypothesized that MMP-9 may be a possible cause of retinal edema and blood–retinal barrier damage in CRVO [33]. In light of this first result obtained that LCN2 increases, we believe that the destructive process forming CRVO may be caused by the inflammatory effect of LCN2 alone and/or triggering of MMP-9 activity. Based on this hypothesis, additional studies involving larger cohorts are necessary to investigate the level of MMP-9 in the aqueous humor of patients with CRVO and to confirm the results of this study.

In conclusion, we have shown for the first time that LCN2 increases in the eye of patients with CRVO. After reperfusion of broad ischemic areas related to CRVO, increased LCN2 levels begin an intense neuroinflammatory process and cause iron accumulation in retinal cells which may create the incendiary progress encountered in CRVO. Additionally, formation of an MMP-9/LCN2 complex promotes a pro-catabolic phenotype and may play an important role in the dreaded complications of CRVO that lead to blindness such as macular edema, macular ischemia, and neovascularization. Nowadays, although VEGF is a target to reduce macular edema in patients with CRVO, based on our data, we propose that LCN2 or MMP-9/LCN2 complex may be potential targets for

CRVO treatment in the future. Additional studies involving larger cohorts are necessary to confirm the results of this study and to show that suppression of MMP-9/LCN2 levels can reduce the complications of CRVO. Such studies will increase our understanding of the pathogenesis of LCN2 and MMP-9/LCN2 complex in CRVO and may provide a basis for therapeutic approaches to the treatment of patients with CRVO.

Compliance with ethical standards

Conflict of interest Authors do not have any financial interest in any products mentioned in this article.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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