

Percentage expression of neutrophil activation marker in the peripheral blood of patients with dry eye disease plus type 2 diabetes

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Background: Dry eye disease (DED) is a disorder of the anterior segment of the eye which is common in patients with type 2 diabetes mellitus (T2DM). It has been reported that symptoms of DED were observed in 60-70% of patients with T2DM. Given that inflammation has a key role in the progression of both T2DM and DED, we believe it is reasonable to study the role of neutrophils in this process.

Purpose: To assess the percentage expression of CD15, a neutrophil activation marker, in the peripheral blood of patients with both DED and T2DM.

Material and Methods: Forty-six patients (92 eyes; mean age, 54.0 ± 8.0 years) with both DED and T2DM were included in this study. There were 19 (40%) women and 27 (60 %) men. Mean diabetes duration was 8.0 ± 6.6 years. All patients had well-compensated diabetes. In addition to a routine examination of the eye, Schirmer I test and tear film break-up time (TBUT) test, they had their corneal fluorescein staining (CFS) scored using the Oxford schema, severities of dry eye graded according to the DEWS II classification and Ocular Surface Disease Index (OSDI) obtained. An immunohistochemical study was employed to assess the expression of CD15, a neutrophil activation marker, in peripheral blood cells of patients.

Results: The mean percentage expression of CD15, a neutrophil activation marker, in the peripheral blood, was 46.7% for 14 patients with T2DM plus DED and punctuate keratopathy, and 28.5% for 32 patients with T2DM plus DED and intact cornea ($p=0.0001$). There was a mild negative correlation between the expression of CD15 and the Schirmer score ($r = -0.32$; $p = 0.032$), and between the former and the TBUT score ($r = -0.34$; $p = 0.019$).

The global prevalence of type 2 diabetes mellitus (T2DM) has increased considerably, and ocular diabetic complications (like dry eye disease (DED), diabetic keratopathy (DK), and diabetic retinopathy (DR)) may result in serious visual impairment and blindness [1, 2].

DED is a disorder of the anterior segment of the eye which is common in patients with T2DM. It has been reported that symptoms of DED were observed in 54-70% of patients with T2DM [3, 4].

Chronic hyperglycemia is the core causative mechanism for DK, and causes the release of various cytokines, chemokines, cell adhesion molecules and other proapoptotic genes [5].

Although it is known that the inflammatory component plays a key role in DED [6], the role of neutrophils in the pathogenesis of the disease is still poorly understood. Increased activity of matrix metalloproteinases [7, 8],

neutrophil elastase [8], myeloperoxidase [7, 8] and Neutrophil Extracellular Traps (NETs) [7, 9, 10] are pathognomonic signs of the presence of neutrophils in the tear fluid of patients with inflammatory ocular surface disorders like DED. The involvement of neutrophils increases with increased severity of DED and associated conditions. It has been reported that NETs play a role in the pathogenesis of severe DED [11, 10], and that the medications for NETs may be helpful in severe DED.

Some experimental and clinical material has been accumulated that allows us to consider the activation of neutrophils as a reaction that, in the presence of inflammation and under certain conditions, may result in their uncontrolled cytotoxicity as well as tissue destruction. The mechanisms are, however, still to be elucidated.

Various proteins are known to be located on the surface of immunocompetent cells, and it is the identification of these proteins that will make it possible to assess the function of these cells. These proteins are named CD markers, have a role as receptors involved in cellular interaction, and are components of signal transduction pathways. The official listing of determinants has identified over 350 individual and unique markers, but we need detailed studies on some of them for understanding pathological processes, particularly those in diabetes. Kuryltsiv and colleagues [12] assessed the expression of CD15, a neutrophil activation marker, and CD95, a marker of apoptosis, in patients with intermediate uveitis, and hypothesized that neutrophils may be responsible for tissue damage in these patients.

Given that inflammation has a key role in the progression of both T2DM and DED, we believe it is reasonable to study the role of neutrophils in this process.

The purpose of this study was to assess the percentage expression of CD15, a neutrophil activation marker, in the peripheral blood of patients with both DED and T2DM.

Material and Methods

Forty-six patients (92 eyes; mean age, 54.0 ± 8.0 years) with both DED and T2DM were included in this study. There were 19 (40%) women and 27 (60 %) men. Mean diabetes duration was 8.0 ± 6.6 years. All patients had well-compensated diabetes, with a mean glucose level of 7.2 ± 0.8 mmol/L. The control group was composed of 26 healthy individuals (mean age, 63.76 ± 6.67 years). Exclusion criteria were connective tissue disorders, history of eye surgery, current use of any ocular medication, and a 7-day or shorter history of acute inflammation of the ocular surface.

In addition to a routine examination of the eye, Schirmer I test and tear film break-up time (TBUT) test, patients had their corneal fluorescein staining (CFS) scored using the Oxford schema, severities of dry eye graded according to the DEWS II classification and Ocular Surface Disease Index (OSDI) obtained [13, 14].

The study followed the ethical standards stated in the Declaration of Helsinki, European Convention on Human Rights and Biomedicine, relevant provisions of the WHO, and Order of the Ministry of Health of Ukraine No.281 of November 1, 2000.

Patients with T2DM were divided into two groups, group 1 (dry eye with punctate keratopathy; 14 patients) and group 2 (dry eye with intact cornea; 32 patients), based on the state of the cornea. Most patients of group 1 had a CFS grade of 1 or 2, whereas patients of group 2 had a CFS grade of 0, using the Oxford schema [13].

Severities of dry eye graded according to the DEWS II classification. Patients of group 1 had moderate (grade 2) DED, whereas patients of group 2, mild (grade 1) DED [14].

Patients of group 2 (T2DM+DED with intact cornea) had an OSDI score corresponding to a dry eye of mild

severity, whereas most patients of group 1 (T2DM+DED with punctate keratopathy), an OSDI score corresponding to a dry eye of moderate severity [13].

An immunohistochemical study using monoclonal antibodies (the peroxidase anti-peroxidase method) [15] was employed to assess the expression of CD15, a neutrophil activation marker, in 46 patients with both DED and T2DM and 26 individuals of the control group. CD15, a neutrophil activation marker, is expressed on mature granulocytes, monocytes, and normal and leukemic myelomonocytes.

In the course of immunohistochemical study, clear neutrophil suspension was obtained and supplemented with monoclonal antibodies specific for CD15. One hundred free neutrophils were evaluated under the microscope, and the percentage of CD15-positive neutrophils was calculated. CD15-positive neutrophils exhibit a dark brown rim of the cytoplasm [15].

Statistica 8 (StatSoft, Tulsa, OK, USA) software was used for statistical analysis. Data are represented as the mean (M) \pm standard error of mean (m) or standard deviation (SD). The Kolmogorov-Smirnov test was used to check for the normality of data distribution. The Student's t-test for independent and dependent samples was applied. Spearman's correlation coefficient (r) was used to assess correlation between the expression of CD15 on neutrophils and the Schirmer score, and between the former and the TBUT score.

Results

The distribution of CD15 expression on peripheral blood neutrophils of patients with both DED and T2DM had two maximum values: neutrophil CD15 expression in 39% of the patients with both DED and T2DM was 10% to 30%, and in another 39% of the patients, 40% to 50% (Fig. 1, Table 1). The mean expression of CD15, a neutrophil activation marker, in the peripheral blood, was 46.7% for T2DM patients with punctate keratopathy, and 28.5% for T2DM patients with intact cornea (the difference was statistically significant), and 12.0 ± 4.0 % for the controls ($p=0.0001$; Table 2).

Table 3 shows Schirmer test and TBUT test scores for DED patients with versus without punctate keratopathy. All patients showed reduced tear production, and mean Schirmer score was 5.6 ± 3.7 mm in 5 minutes for those with punctate keratopathy versus 9.5 ± 2.8 mm in 5 minutes for those with intact cornea ($p=0.0004$; Table 3). Mean tear film break-up time was 7.2 ± 2.5 sec for patients with punctate keratopathy versus 8.9 ± 2.3 sec for those with intact cornea ($p=0.0297$) (Table 3).

There was a negative correlation between the expression of CD15 on neutrophils and the Schirmer score ($r = -0.32$; $p = 0.032$), and between the expression of CD15 on neutrophils and the TBUT score ($r = -0.34$; $p = 0.019$).

An OSDI score > 15 was present in all patients, indicating the presence of dry eye symptoms. In addition, mean OSDI score was 17.0 ± 2.0 , corresponding to a dry

eye of mild severity, for patients of group 2 (T2DM plus DED) versus 28.0 ± 2.0 , corresponding to a dry eye of moderate severity, for patients of group 1 (T2DM plus punctate keratopathy).

We found that the percentage expression of CD15 on neutrophils did not depend on patient's gender, type of treatment (blood sugar reducing tablets ($p=0.40$) or insulin injections ($p=0.39$)), age ($p=0.23$), blood sugar level ($p=0.19$), or diabetes duration ($p=0.92$).

Discussion

Recent studies have demonstrated that circulating neutrophils may perform various functions like phagocytosis and protein synthesis, and be involved in oxidative metabolism. Until recently, there were few studies on phenotypic differences in neutrophils, and it was the detection of highly specific markers of neutrophils that enabled to divide this subpopulation of cells into subgroups. In addition, although neutrophils were believed to have only an antimicrobial function, studies have demonstrated that they have various functional responses that go beyond just elimination of microorganisms [16]. Various functional responses of neutrophils are induced by activation of transcription and changes in the expression patterns and activity of the superficial molecules. These phenotypic changes are usually observed only in the subgroup of neutrophils exhibiting heterogeneous phenotypes.

Aside from a protective function, neutrophils have been shown to have an activated state, which, under certain conditions, may result in additional tissue damage. The release of cytotoxic products from neutrophil granules leads to tissue disintegration [17].

Enough material has been accumulated that allows us to consider the activation of neutrophils as a reaction that, under certain conditions, may result in their uncontrolled cytotoxicity as well as tissue destruction [17].

It should be noted that activated neutrophils circulating in blood are very different in behavior from those in tissues. Wedmore and Williams [18] demonstrated that activated neutrophils have a disruptive effect on the integrity of the epithelium and cause increased permeability and dissociation of endothelial cell-to-cell junction proteins. The interaction of circulating neutrophils with activated endothelium is a central phenomenon in inflammation, and involves various molecular events associated with neutrophil adhesion and migration as well as impaired endothelial permeability. Neutrophils must be stimulated by inflammatory mediators and by adhesion surfaces for the initiation of cytotoxic activity [19].

Excessive neutrophil-endothelial cell adhesion may result in endothelial damage by proteases and oxidants [20].

Endothelial dysfunction and increased levels of anti-inflammatory cytokines may result in leukostasis and increased interaction of leukocytes with epithelial cells.

Subsequently to neutrophil activation, there is the release of elastase and myeloperoxidase from neutrophil

granule, leading to corneal damage. It has been demonstrated in animal models and in human disease that the prevention of or reduction in leukocyte-endothelial cell adhesion frequently resulted in significantly reduced microcirculatory dysfunction and parenchymal cell dysfunction [4].

In addition, it has been hypothesized that ocular surface infiltration with T cells (particularly, Th1 and Th17) is implicated in damage to ocular surface epithelial cells and plays a major role in the development of dry eye disease [21, 22].

We have previously assessed the activity of oxidative reductive enzymes and glutathione levels in the cornea, conjunctiva and tear fluid in experimental streptozotocin-induced diabetes. We found that the activity of oxidative reductive enzymes in the cornea of animals with acute conjunctivitis in the presence of streptozotocin-induced diabetes was substantially decreased compared to animals with conjunctivitis induced in the absence of experimental diabetes. In addition, under the above conditions, the activity of oxidoreductases (lactate dehydrogenases, malate dehydrogenases and glucose-6-phosphate dehydrogenases) in the tear fluid is significantly increased, indicating impaired stability of cell membrane structures [23, 24]. Moreover, it was noted that, the development of inflammation in the conjunctiva under hyperglycemic conditions results in a more severe impairment in glutathione redox status (particularly, a reduction in reduced glutathione and increase in oxidized glutathione) than in conjunctival changes in non-diabetic animals [25-27]. The results obtained indicated an impairment of the antioxidant system and damage to lysosomal membranes of epithelial cells in experimental diabetes, with these system and membranes playing a major role in homeostasis of the ocular surface.

We were the first to assess the expression of CD15, a neutrophil activation marker, in peripheral blood cells of patients with both DED and T2DM.

Given the fact that activated neutrophils have a role in the development of changes (i.e., triggering of immune inflammation) in the ocular surface, it would be reasonable to consider integrating anti-neutrophil therapy (aiming to reduce the release of inflammatory mediators) use as an adjunct in T2DM management.

The use of anti-inflammatory (anti-neutrophil) therapy will reduce capillary permeability (and, consequently, reduce the exudative component of inflammation); stabilize lysosomal membranes and reduce lysosomal enzyme secretion (thus reducing tissue alteration); and inhibit the synthesis of inflammatory mediators [18, 19].

The differences that we found in the expression of CD15, a neutrophil activation marker, in peripheral blood cells, between the two groups (the group of patients with DED plus T2DM and the presence of punctate keratopathy, and the group of those without punctate keratopathy), suppose that CD15 may be a subclinical predecessor of immune inflammation in the cornea.

The data obtained suppose that activated neutrophils may trigger immune inflammation resulting in corneal damage in patients with T2DM due to the release of pro-inflammatory cytokines.

Understanding of the mechanisms and the role of neutrophils in the pathological processes in the cornea in patients with DED plus T2DM suggests the need for the use of anti-leukocyte agents to prevent keratopathy progression.

Therefore, further studies are required (a) to determine changes in the phenotype of neutrophils in DED for improved understanding of their role in abnormal regulation of the ocular surface and (b) to assess their role as biomarkers of the disease.

Conclusion

First, we found that, the mean expression of CD15, a neutrophil activation marker, in the peripheral blood, was 46.7% for patients with T2DM plus DED and punctuate keratopathy, and 28.5% for patients with T2DM plus DED and intact cornea ($p=0.0001$).

Second, there was a negative correlation between the expression of CD15, a neutrophil activation marker, and the Schirmer score ($r = -0.32$; $p = 0.032$), and between the former and the tear film break-up time (TBUT) score ($r = -0.34$; $p = 0.019$).

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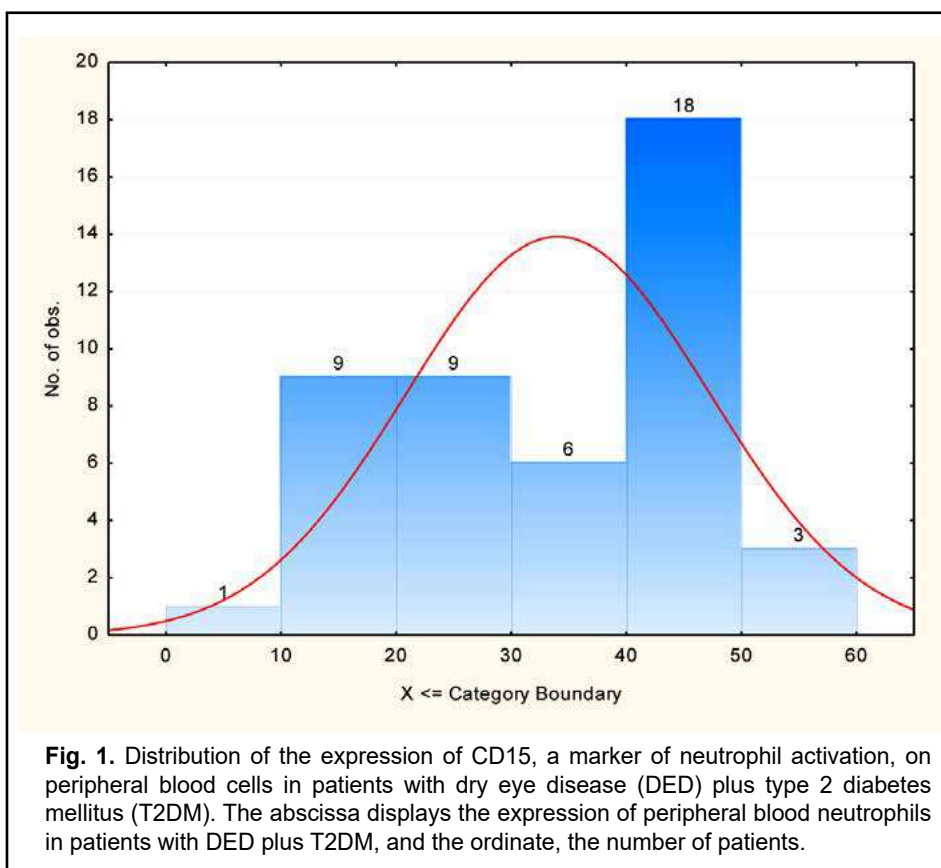


Table 1. Percentage expression of CD15, a neutrophil activation marker, on peripheral blood cells in patients with dry eye disease (DED) plus type 2 diabetes mellitus (T2DM)

	n	M	p	Median	Min	Max	SD
Percentage expression of CD15, a neutrophil activation marker	46	34.1	1.00	38.5	10.0	56.0	13.2

Note: n, number of patients; p, probability of significant difference; M, mean value; Median, median value; Min, minimum value; Max, maximum value; SD, standard deviation

Table 2. Percentage expression of CD15, a neutrophil activation marker, on peripheral blood cells in patients with dry eye disease (DED) plus type 2 diabetes mellitus (T2DM) with and without punctate keratopathy

Characteristic	Control group, n=26	Keratopathy (group 2), n=14	No keratopathy (group 3), n=32	t	p1-2	p1-3	p2-3
Percentage expression of CD15, a neutrophil activation marker	12.0±4.0	46.7±4.25	28.5±11.9	5.55	0.0000	0.0000	0.0001

Note: n, number of patients; p1-2, probability of significant difference between the control group and the group with T2DM plus keratopathy; p1-3, probability of significant difference between the control group and the group with T2DM without keratopathy; p2-3, probability of significant difference between the the group with T2DM plus keratopathy and the group with T2DM without keratopathy; t, Student's coefficient.

Table 3. Schirmer score (M±SD) and tear film break-up time (TBUT) test score (M±SD) in patients with dry eye disease (DED) plus type 2 diabetes mellitus (T2DM) with and without punctate keratopathy

	Keratopathy, n=14	No keratopathy, n =32	t	P
Schirmer score, mm/5 min	5.6 ± 3.7	9.5 ± 2.8	3.8	0.0004
TBUT test score	7.2 ± 2.5	8.9 ± 2.3	2.2	0.0297

Note: n, number of patients; p, probability of significant difference; M, mean value; t, Student's coefficient; SD, standard deviation