Diabetic retinopathy: proteomic approaches to help the differential diagnosis and to understand the underlying molecular mechanisms

Éva Csősz[1], Eszter Deák,[1,2], Gergő Kalló[1], Adrienne Csutak[2], József Tőzsér[1]

[1] Proteomics Core Facility, Department of Biochemistry and Molecular Biology, [2] Department of Ophthalmology, Faculty of Medicine, University of Debrecen.

Corresponding author: József Tőzsér

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Diabetes, diabetic retinopathy

Diabetes Mellitus (DM) has become the epidemic of the 21st century, the current worldwide prevalence of the disease is 2-6 % [1]. DM is described as a metabolic disorder of multiple etiology, characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [2]. Insufficient insulin action arises either due to the damage of the producing pancreatic cells (insulin deficiency), or because the insulin produced cannot work properly (insulin resistance), or due to the two mechanisms together. DM is defined by the World Health Organization (WHO) as a fasting venous plasma glucose concentration of equal to or greater than 7.0 mmol/L or venous plasma concentration of equal or greater than 11.1 mmol/L, 2 hours after oral intake of 75 g glucose [3].

The main problem with DM is the occurrence of metabolic, vascular and neurological complications [4,5]. The effects of the disease include long–term damage, dysfunction and failure of various organs [6]. Early detection and treatment of the disease can decrease the risk of developing complications. Keeping the blood sugar level close to normal can dramatically reduce the risk of several complications.

Patients with diabetes are at high risk (approximately 90%) of developing diabetic retinopathy (DR), which is the most common complication of diabetes mellitus [7]. Diabetic retinopathy is currently the leading cause of blindness in the economically active population in developed countries [8]. This condition is of vascular origin, and is characterized by signs of retinal ischemia (microaneurysms, hemorrhages, exudates, intraretinal microvascular abnormalities, abnormalities in the venous caliber and neovascularization) as well as signs of increased vascular permeability [9]. This progresses from mild non-proliferative disease to moderate or severe non-proliferative retinopathy (NPDR), and can finally lead to proliferative (PDR) disease [10].

Inflammation and neurodegeneration as possible causes of DR

Diabetic retinopathy is also categorized as a chronic low-level inflammatory disease [11–13]. The appearance and the severity of the DR is correlated with the duration of diabetes and poor blood glucose level management [14]. The high blood glucose level promotes the accumulation of the advanced glycation end products [11] and leads to the stimulation of monocytes and macrophages [12]. It was shown that the level of circulating TNFα is significantly higher in the serum of patients having PDR than in the serum of patients having NPDR [13]. The level of proinflammatory cytokines such as MCP-1, IL6, TNFα and of MMP-9, inducible NOS, ICAM-1 was shown to be increased in the eye of diabetic patients [15], moreover, the balance between the pro- and anti-angiogenic cytokines is shifted leading to elevated pro-angiogenic cytokine levels in patients with retinopathy [16].

The uncontrolled serum glucose level, the mitochondrial and extracellular reactive oxygen species (ROS) are toxic to endothelial cells and neurons, thus contribute to their death by initiating apoptosis [17,18]. The retinal neurons and vascular cells depend on insulin receptor activity, and the reduced insulin action leads to neurodegeneration. Hence, insulin

signaling appears to provide neurotrophic actions in the retina, and retinopathy may results in part from neurotrophin deficiency [19].

Screening in the diagnosis of DR

As timely diagnosis and therapy can significantly slow down the development and progression of DR, screening and appropriate follow-up of patients with diabetes is strongly desired [7]. Screening can be carried out by direct and indirect ophthalmoscopy or by using the increasingly more popular photographic methods [20]. The neovascularization and increased vascular permeability in the retina are visible and recordable symptoms as microaneurysms and macula edema and their detection could substantially contribute to the differential diagnosis of the DR [21,22].

Photographic methods currently use digital images with subsequent grading by trained individuals. Regular DR screening is centralized in several developed countries due to cost-efficiency and quality control issues [23]. More than 50% of screened patients in general, are negative (no-DR) and are advised to rescreen in 1 year [24]. All the available DR screening methods rely on the involvement of human resources which contributes greatly to the costs of these methods. In order to improve scalability and cost-effectiveness, several research groups are working on developing automated image analysis technologies [25]. Preliminary results are promising, sensitivity and specificity indicators of automated systems are close to that of human graders [26,27]. A hallmark of DR is the appearance of microaneurysms (MAs). As these out-pouches occur on small blood vessels, most of the image processing based screening methods concentrate on the detection of this type of lesions [28]. Besides computer-based image processing other methods can also support DR screening. Tear fluid proteomics is such a possible novel tool for population screening, which is based on the pre-screening of a large number of patients and uses human graders only in positive or ambiguous cases [29].

In order to increase the sensitivity and specificity values of diabetic retinopathy screening method, our research group used a MA detector combined with tear fluid proteomics based methodologies in one single system [30].

Proteomics methods applied for DR diagnosis

The study of proteins differentially expressed in the eyes of patients with DR may provide biomarkers which can help the diagnosis. Both gel-based and gel-free approaches are used to gain information on the proteome-wide changes and to identify individual proteins which can help to understand the patomechanism of DR and to be used as potential biomarkers.

Two dimensional electrophoresis (2DE) is a versatile gel-based technique making possible the qualitative and quantitative analysis of proteins [31]. In the first dimension the complex protein mixture is separated based on the charge (pI) followed by a separation based on the size in the second dimension. The gel image analysis can visualize the spots showing differential expression between samples/conditions and the mass spectrometry-based protein identification can identify the proteins present in the selected spots [32]. The combination of 2DE with protein identification can give information about the protein-level changes characteristic for different conditions [33]. A more sophisticated form of 2DE is the differential gel electrophoresis (DIGE) where the samples originating from different groups are labelled with fluorescent dyes and are mixed and processed together. After the implementation of 2DE the gel is scanned with laser scanner and the differential image characteristic for each fluorescent dye (sample) can be visualized [34] thus saving time and reducing the workload.

The gel-free approaches include usually liquid chromatographic (LC) separation of proteins preceded in many cases by other off-line separation techniques such as solid-phase extraction or strong cation exchange [35,36]. In typical conditions the LC is coupled on-line with mass

spectrometer and the proteins/peptides eluted from analytical column are directly injected into the mass spectrometer for identification and/or quantification [37].

Quantitative proteomics is able to monitor the relative or absolute changes in individual protein levels in various conditions. Chemical labeling with iTRAQ or TMT makes possible the labeling of up to 8 or 10 different samples with an isobaric label [38–43]. The label is constructed in that way that the m/z values of the peptides originating from different samples (precursor ions) are the same but during the fragmentation the labeling group is released and the rest of the peptide can be used for MS/MS based protein identification. Based on the area-under the curve (AUC) values of the labeling groups the relative amounts of the proteins in the studied samples can be calculated [44]. One drawback of the chemical labeling can be the efficiency of the labeling reaction and also the presence of the side-products which can make the evaluation of the results problematic. Targeted proteomics based on selected/multiple reaction monitoring (SRM/MRM) has the benefit of high sensitivity and specificity making possible the simultaneous quantification of multiple proteins [45,46]. In this setup two restrictions are applied mainly to triple quadrupole-containing mass spectrometers. In the first quadrupole only the ions with the specified Q1 m/z value are stabilized and these ions can arrive to the second quadrupole where the fragmentation happens. In the third quadrupole the second restriction is applied; fragments only with the specified Q3 m/z values are stabilized and can reach the detector. The AUC of the recorded peaks are proportional with the amount of samples introduced into the mass spectrometer. According to the gold standard stabile isotope labeled counterparts of the studied peptides are added in known amounts to the sample and are used both as a qualitative and quantitative reference throughout the experiments [47]. This method does not imply any labeling but prior information is needed for assay design.

The other widely used quantification technique is the so called label-free quantification. In this case the AUC of the precursor ion or the number of the MS/MS events are used for quantification [48]. One limitation of this method can be that it can be applied only on mass spectrometers providing high accuracy and high scanning rate [49].

As the number of laboratories having better performing mass spectrometers increases the parallel reaction monitoring (PRM) and SWATH techniques can provide even more qualitative and quantitative data [50–52] on proteome changes in different conditions and can provide valuable information on biomarker studies.

The most logical material to be studied is retina, the site of DR complications and the examination of retinal proteins can be considered as a direct way of identifying the occurrence and development of DR. Membrane samples from PDR patients and patients with idiopathic epiretinal membranes (ERMs) were analysed with LC-MS. A total of 225 and 154 proteins were found in the PDR and ERM groups, respectively; 123 being present in both groups, and 102 proteins were unique to PDR and 31 to ERM groups. Periostin as a candidate marker for PDR was chosen and the proteomics result were confirmed by RT-PCR [53]. Further research on the retina proteome has been performed using animal models. Retinas of diabetic rats treated with grape-seed extract (GSPE) were analyzed using 2-DE and mass spectrometry-based protein identification. A total of 18 proteins were found to be differentially expressed in diabetic rats compared with normal rats. The level of aB-crystallin, bA4-crystallin, aA-crystallin, ubiquitin carboxy-terminal hydrolase L1, G protein b1, Ig-2A and pyruvate kinase normalized following treatment with GSPE [54]. The authors hypothesized that the downregulated proteins might play key roles in the development of and recovery from DR [54]. In another study in mice members of crystallin family were found to be upregulated, and the level of each crystallin changed during the course of diabetes treatment [55]. Both systemic and local insulin treatments reversed the upregulation of crystallins, therefore the

authors suggested that insulin treatment could reduce the level of apoptosis observed in DR and noted the possible role that crystallins may play in DR. The VEGF responsible for neovascularization was detected in high amounts in the retina of diabetic rats and emerged as a possible therapeutic target in DR treatment by intravitreal application of anti-VEGF agents [56]

Besides retina, another sample source which is subjected to extensive studies is the vitreous humour which is in close proximity to the retina and may reflect the retinal changes. The vitreous humour is a transparent, gelatinous mass making up about 80% of the volume of the eyeball and is located between the lens and the retina. It plays an important role in providing metabolic nutrient requirements of the lens, coordinating eye growth and providing support to the retina. Most of the proteins being identified in vitreous humour so far, such as albumin, transferrin, apolipoprotein, IgG, transthyretin [57,58], are also found in serum [59]. The vitreous contains matrix metalloproteinases (MMPs) [60], which are zinc-dependent enzymes maintaining the structure and function of the extracellular matrix [61]. In a comparative proteomics study the protein composition of retina and vitreous humour was analysed showing proteins which functionally interact with each other to control oxidative stress, immune reactions and the exchange of intracellular proteins between the retina and vitreous [62]. It was demonstrated that the total protein content reported for the vitreous of patients with DR is higher than in the non-diabetic and control samples [63].

Most proteomic studies have been conducted on vitreous fluid obtained from diabetic patients undergoing surgery for PDR. Vitreous humour proteins from PDR patients and non-diabetic patients with macular holes was studied using DIGE followed by mass spectrometry. The upregulation of zinc-α2-glycoprotein, apolipoprotein (APO) A1, APOH, fibrinogen A, complement factors C3, C4b, C9 and factor B and downregulation of pigment epithelial-derived factor, interstitial retinol-binding protein and inter-α trypsin inhibitor heavy chain was

observed [64]. Other researchers also observed elevated APOA1 and APOH levels in PDR patients which may lead to nutritional disorders and induce disease [65].

Another widely studied sample is the aqueous humour, the fluid in the anterior and posterior chamber of the eye produced by the pigmented and non-pigmented epithelium of the ciliary body. After secretion into the posterior chamber, the aqueous circulates through the pupil into the anterior chamber. In comparison to serum, aqueous humour has a much lower protein concentration (approximately 0.2-0.5 mg/mL) [66] creating a challenge for proteomic studies. Nevertheless, plasma proteins like albumin, globulins [67], and transferrin [68] are present in the aqueous humour [69]. A study comparing the aqueous humour proteins between DR and control groups shown the upregulation of APOA1, serotransferrin, keratin type I cytoskeletal 9, keratin type I cytoskeletal 10, growth factor receptor-bound protein 10, brain-specific angiogenesis inhibitor 1-associated protein 2 and cystathionine b-synthase and downregulation of matrix metalloproteinase 13, podocan and selenoprotein P in the DR group [70].

The retina, vitreous and aqueous humours can give reliable information regarding DR pathogenesis but they can be collected by invasive methods during different forms of surgery carried out in most of the cases to manage the complications of the DR, thus they cannot be widely used for diagnostic purposes. The non-invasive character of the biomarkers is critical in case of DR diagnosis as far as screening methods are available and are used as a gold standard in DR diagnosis. Methods which are able to improve the screening performance are required, preferably methods which can be carried out by general health care practitioners.

Tear proteomics as a tool in DR specific biomarker studies

The necessity for other modalities which are able to improve the sensitivity and specificity of DR diagnosis has turned research toward non-invasively collected samples and

led to the intensive studies on the identification of tear biomarkers which alone or in combination with other methods can be used for screening purposes. The protein composition of tear fluid has been investigated by numerous research groups and more than 1500 proteins have been identified so far [71–73]. Concerning the fact that tear is not in direct contact with retina the utilization of tear as a source of biomarkers in DR can be questionable. Taking into account that in DR there are problems with the alterations of the vasculature which in turn lead to retinal changes (ref), the altered blood flow can modulate the composition of the tear secreted by lacrimal gland and in this way the tear may reflect the changes of the retina even if there is no direct contact between them. It is not known if there is any direct, cause-effect relation between retinal processes and the change in the composition of the tear proteins but correlation between PDR progression observed and tear protein profile changes were demonstrated. Some of the proteins were identified as possible biomarkers for diabetic retinopathy, even if they may have not directly been implicated in the patomechanism of DR. Significantly elevated levels of nerve growth factor, APOA1, lipocalin 1, lactotransferrin, lacritin, lysozyme C, lipophilin A and immunoglobulin lambda chain have been observed in PDR, while reduced level of lipocalin-1, hsp27, β_2-microglobulin and increased levels of endothelin and neuron-specific enolase were shown in tears of patients with NPDR [38,74–77]. There is high need for the identification of useful biomarkers for diagnosis and monitoring the progression of the DR, proteins which are able to distinguish between the patients without retinopathy, with non-proliferative and with proliferative stages of DR. Different sets of proteins were identified as potential biomarkers in retina, vitreous, aqueous humour and tears, the only overlap is the presence of APOA1 in vitreous, aqueous humour and tears (Table 1). The so far identified potential biomarkers are mainly highly abundant tear proteins which may not have direct implication in the pathogenesis of DR; the alterations is their levels can rather be the consequences of the disease. More studies with high sample size

are needed to validate these potential biomarkers. There are some factors which make the comparison of the results of biomarker studies difficult: (1) the number of the patients enrolled, (2) the groups selected for study, (3) the type of tears used for analysis, (4) the applications of different tear collection techniques and finally (5) the different proteomic strategies applied. Practically there are four different possible groups to study: i) healthy, normal controls without diabetes, ii) diabetic persons without retinopathy, iii) patients with non-proliferative or early stage and iv) patients with advanced or proliferative stage of retinopathy. In the different studies various combinations of the patients groups are used. In general, the number of patients enrolled into each group is below 20; there are fewer studies carried out on higher number of patients [38,74]. Regarding the sample type used for biomarker studies, besides tears different biological fluids such as serum [77] and vitreous [78] were administrated. Considering the tears, the different sample collection techniques applied may include another level of variations. Studies carried out may include the unstimulated basal tear or the reflex tear which has an altered composition as a response to injury or tear production stimulation such as application of intranasal ethanol [79]. It seems that the most reliable method would be the basal tear collection with a clean glass capillary without disturbing the eye in any way [80]. Another widely used method in routine clinical applications is with Shirmer's strip which uses a strip of absorbant paper placed into the lower eyelid provoking a low-grade irritation practically making impossible the collection of basal tears. Comparing the proteins identified from tears using either capillary or Shirmer's strip have revealed that the number of proteins identified using Shirmer's strip for tears collection is higher [81] highlighting the more invasive feature of the method. Also it should be noted that the progression of diabetes is associated with dry eye disease, which is more prevalent in patients with PDR [82]. In these cases the collection of basal tears with glass capillary can be very problematic or even impossible [83].

Tear proteins have been studied using a wide array of protein detection methods as well as proteomics techniques from antibody-based ELISA and Western blot to the more sophisticated mass spectrometry methods [83]. Electrophoresis [84], protein arrays [85] and various mass spectrometry related techniques [83,86] have been applied. Various forms of chemical labeling [39,43], SRM/MRM-based targeted proteomics and label free quantification have been used for tear studies but no PRM or SWATH analyses have been carried out so far [87,88].

These more advanced quantitative proteomics techniques making possible more sensitive analyses are extremely important in tear studies because the assays with lower sensitivity usually can give information only about the changes of the abundant tear proteins. The presence of the highly abundant proteins can be a limitation in the tear proteome analyses but in many cases the changes of the abundant tear proteins can have diagnostic importance as well [89,90].

The chemical barrier of the tears as a possible source for biomarkers

At those sites where our body comes in contact with potential pathogens, a well-defined chemical barrier exists. The chemical barrier provides a passive protection against the infections by diluting the pathogens, as well as an active one by carrying the secreted antibacterial and antiviral proteins that inhibit bacterial growth [91]. Regarding the protein composition of the chemical barrier it appears that the highly abundant proteins have protective properties. In case of sweat and tears it was demonstrated that the proteins comprising more than 90 % of the secreted proteins have a role in defense [73,92].

In good agreement with the above-mentioned roles, the functional analysis of the tear proteome revealed that the major tear proteins such as lactoferrin, lysozyme C, lipocalin etc., are involved in the immune and inflammatory processes, oxidative stress response and

defense against pathogens [71,73,83,86]. It has been shown that lactotransferrin is active against microbes and parasites and it has been implicated in the protection against cancer [93]. Because of its iron sequestration activity, lactotransferrin has an important role in the prevention of bacterial colonization. The level of tear lactotransferrin reflects the lacrimal gland function [94] which can have diagnostic value in several pathological conditions [95,96]. Lysozyme-C is a hydrolytic enzyme with muraminidase activity required for the peptidolycan degradation of the bacterial cell wall [93]. Besides its antimicrobial activity, lysozyme-C has various functions including activity against several fungi [97] and protection against HIV infection [98]. A recent study revealed 32 protease inhibitors in tears such as cystatins, lipocalin-1 and -2, α_2-macroglobulin and α_2-HS-glycoprotein, etc. [71]. This large number of protease inhibitors indicates that the chemical barrier of the eye may have an active defense mechanism against microbial proteases and may limit the cell damage caused by the proteases secreted into the tear fluid.

Other important but low abundance components of the chemical barrier are the proteins and peptides belonging to the antimicrobial and immunmodulatory peptide (AMP) family being part of the innate immune system and forming the chemical barrier in the first line host defense. The chemical barrier contains mostly constitutively expressed AMPs, while some of them show inducible feature upon pathogenic or other stimuli. The tear fluid contains several prototypic AMPs such as defensins, dermcidin and LL-37 cathelicidin [73,83,99,100]. Defensins are small cationic peptides produced by epithelial cells with cysteine rich sequences forming a very stable 3D structure [101]. The two subfamilies of defensins (α- and β-defensins) show broad antimicrobial as well as antifungal and antiviral activities [93,102]. While some defensins, like β-defensin 1 show constitutive expression pattern, other members of this family found to be induced upon pathogenic or inflammatory stimulations [103]. Using an SRM-based targeted proteomics method to study the beta defensin 1, 2, 3 and 4 levels in

tear it was demonstrated that the level of β-defensin 2 was the lowest, the level of β-defensin 3 was the highest and the levels of β-defensin 1 and β-defensin 4 were very similar to each other [104]. LL-37 cathelicidin is an α-helix type AMP of the tear fluid mainly produced by the corneal epithelial cells. Similar to the defensins, cathelicidin exerts various antimicrobial [100] and immunmodulatory functions [93]. In addition, it has an important role in re-epithelialization during wound healing [105]. Dermcidin, the main skin AMP present in tears exerts broad spectrum antimicrobial activity [106–108]. The mRNA expression of dermcidin has been detected in corneal and conjunctival epithelial cells [109]. As far as in the chemical barrier different concentrations of the various host defense proteins can be observed, practically an AMP cocktail is present which implies interactions between the AMP molecules. It has been shown that β-defensin 4 functions as a synergistic partner of lysozyme-C and together with β-defensin 3 exert antimicrobial and immunmodulatory activities [110]. Another synergistic partner of lysozyme-C was found to be lactotransferrin [111].

The composition of the AMP cocktail changes constantly, implying both qualitative and quantitative changes of the cocktail components, in order to adapt to various conditions. By analyzing some of the components of the chemical barrier using SRM-based targeted proteomic assay our group could demonstrate the changes of the chemical barrier composition related to the progression of diabetic retinopathy.

Most probably the composition of the chemical barrier is characteristic to the stimulus to which the organism has to adapt or to the pathological condition causing the alteration of the chemical barrier leading to the characteristic ocular symptoms. Thus the changes of the composition of the host defense proteins at the ocular surface as a response to pathological conditions may provide a feasible source for biomarker studies.

Ocular wound healing

The alterations of the chemical barrier in DR can have consequences on the physiological processes mediated by the components of the chemical barrier. It is well known that in the diabetic patients a deregulation of corneal repair mechanisms may be present accompanied by delayed wound healing, recurrent erosions and ulcers [112]. The so called diabetic keratopathy is associated with wound healing complications which may arise after vitreoretinal surgeries used for the management of the proliferative diabetic retinopathy, where oedematous and cloudy corneal epithelium, often manually removed to restore clarity, results postoperatively in a poorly healing corneal epithelial surface [113]. Similarly to the skin-related problems in diabetic foot the changes of the composition of the chemical barrier of tears in the different stages of the diabetic retinopathy may lead to improper defense resulting in recurrent infections and impaired wound healing (Figure 1) [114].

The process of wound healing involves a complex, dynamic system with cell-cell and cell-matrix interactions in response to injury. This entire process has four sequential, overlapping phases: hemostasis, inflammation, proliferation or tissue formation and remodeling as well as scar formation. Normally, after the injury, the hemostatic cascade system is activated and controlled by activators and inhibitors [115]. The activated platelets play a key role in this step, they release several chemicals (thrombin, thromboxane A2, serotonin, platelet activating factor, etc.), cytokines (transforming growth factor [TGF-β], interleukin [IL-1,IL-8], macrophage inflammatory protein [MIP-1 and -2], etc.) and growth factors (platelet derived growth factor [PDGF], vascular endothelial growth factor [VEGF], insulin-like growth factor [IGF-1], connective tissue growth factor [CTGF], etc.) to promote the clotting and the further wound healing phases [116]. In the next step, the inflammatory cells, like the neutrophils and the monocytes, are activated by the chemo active agents and migrated to sites of tissue injury. Neutrophils infiltrate the wound and clear it up from debris and as an effect of complement system they enhance the opsonization of bacteria. Monocytes,

after transforming to macrophages, help contributing to tissue debridement and phagocytosis but they also release growth factors (PDGF, TGF-β, epidermal growth factor [EGF], fibroblast growth factor [FGF], etc.) to facilitate the migration of endothelial cells, fibroblasts and keratinocytes to the wound cavity. Another essential inflammatory component is the T-lymphocytes, which also release cytokines (PDGF, TGF-β, IL-4, interferon [IFN-γ], etc.) to stimulate fibroblasts, macrophages and endothelial cell function [117]. During the proliferation phase, the cytokines, growth factors and proangiogenic agents (ANG-1 and -2) released by inflammatory cells initiate the synthesis of extracellular matrix (ECM) components (different types of collagens and glycosaminoglycan) and the neovascularization or angiogenesis [118]. Finally, the remodeling and the formation of scar tissue through the ECM stabilization take place. Blood vessels regress over time, and fibroblasts induce crosslinking of collagen type I and elastin, which leads to a dense scar tissue formation [119].

Defects or alterations in any phase of wound healing can contribute to the pathology of various diseases and cause delayed healing, increased pain and risk of infections, as well as decreased quality of life. A lot of systemic factors can influence the wound healing process, for example, the age, gender, hormonal status, stress, alcoholism, smoking, medication use, inadequate nutrition, obesity and diabetes [120]. Using wound healing models it was shown that the overexpression of cathepsin S and matrix metalloproteinase MMP-10 down-regulate protein kinase B and inhibit wound healing, while c-Met is required; up-regulation of c-Met increases corneal wound healing [112,121,122].

The altered or delayed healing is related to inflammation in aged populations [123], and these alterations can result in reductions in angiogenesis and remodeling of the wound site. Strangely, in elderly individuals while the inflammation seems to be increased, and simultaneously the macrophage function is reduced [124].

Several studies provided evidence that the deregulation of wound healing is due to aberrant inflammation associated with obesity [125] and diabetes [126]. Several research groups reported, that IL-1β, IL- 6, IL-8, IP-10, MCP-1 and TNFα were found to be increased in chronic wounds [127] and also during retinopathy progression [128–130]. Furthermore, impaired healing is associated with deregulation of collagen turnover, including increased MMP; especially MMP-2 and MMP-7 [131,132] and decreased tissue inhibitors of metalloproteinase (TIMP) activity deregulating the MMP-TIMP balance [133] as well as decrease in angiogenesis, granulation tissue formation, and collagen deposition [134].

There are some potential targets for therapeutic intervention in diabetic wound healing, and identifying the key steps of the inflammatory process contributing to tissue destruction will help to control ocular wound healing and its complications during diabetic retinopathy.

Conclusion

In the last decade, several studies focused on the biomarker and therapeutic molecule identification. Tear is an excellent candidate for biomarker discoveries as far as it can be collected easily and it is all the time accessible. The so-called “omics” disciplines (genomics, proteomics, metabolomics, etc.) help to understand the global platform of molecular events in diabetic retinopathy and in wound healing. By searching the up- and down-regulated proteins in tears can help to understand the pathophysiology of the eye complications related to hyperglycemia an may improve the quality of life of diabetic patients. For this more standardized studies carried out on larger sample size are needed. Having a better understanding on corneal wound healing and microvasculature changes in diabetes may lead to the discovery of better biomarkers for diagnostic and prognostic purposes and to identification of therapeutic target molecules which can be used for drug development.

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