Chapter 10

A Potential Mechanism for Diabetic Wound Healing: Cutaneous Environmental Disorders

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Abstract

Diabetes mellitus is a chronic multi-organ metabolic disorder caused by a combination of environmental and genetic factors. Diabetic complications are considered to be multifactorial with increasing evidence that one of the major pathways involved in the progression of both microvascular and macrovascular diseases is the biochemical process of advanced glycation.

We will combine in vitro and in vivo studies and other related literatures to discuss the role of advanced glycation end products (AGEs), which may exert deleterious effects in diabetes. Dr Shuliang Lu puts forward the theory of 'cutaneous environmental disorders' mediated by AGEs. The receptor for advanced glycation end products (RAGE) was first described as a signal transduction receptor for AGEs. Recent discoveries regarding AGEs-RAGE interactions expanded our understanding of the mechanisms by which RAGE evoked pathological consequences.

In this chapter, we report on the biology of AGEs, AGEs and wound healing, as well as address current strategies to interrupt the formation of AGEs and underscore strategies by which antagonism of RAGE and AGEs-RAGE crosslinks may be realized.

Keywords: diabetic wound healing, advanced glycated end products, RAGE, measurement, treatment

1. Introduction

Diabetes mellitus is characterized by chronic hyperglycemia and an altered cellular homeostasis, which lead to diffuse vascular damage and multi-organ dysfunction. Diabetic patients

risk both micro- and macro-vascular complications: the former result from damage to retinal, renal, and neural tissues, which is the cause of blindness, end-stage renal failure, and non-traumatic lower limb amputation, respectively [1]. Here, we will focus on diabetic wound. Impaired wound healing is associated with increased morbidity and mortality in diabetes mellitus. The majority of non-healing wounds often lead to amputation, increasing the direct costs of their care, rehabilitation, and lost productivity [2].

According to a national survey, the prevalence of chronic cutaneous wounds among hospitalized patients was 1.7% in China. The leading causes were diabetes (31.3% men, 35.3% women) and trauma (26.4% men, 19.2% women). Therefore, diabetes has recently become the leading cause of chronic cutaneous wounds in China [3]. In Shuliang Lu's study, it was indicated that new diabetic foot ulcers were already in poor condition when patients first visited the diabetic foot clinic. Concomitantly, patients had worse health-related quality of life compared with the general population [4].

Several mechanisms have played a role in this condition, such as neuropathy, peripheral arterial disease, biomechanical factors, infection, and wound healing. Brownlee identifies the production of reactive oxygen species (ROS) as the unifying mechanism behind the main pathological pathways triggered by hyperglycemia, one of which leads to the formation of heterogeneous moieties called advanced glycation end products (AGEs) via non-enzymatic glycation and glycoxidation processes [5]. AGEs affect the wound healing process either directly by their interference with various components involved or indirectly through their association with diabetic neuropathy or angiopathy [6, 7]. In addition, RAGE was discovered as a receptor for AGEs, such as carboxymethyl lysine (CML) [8]. RAGE has been postulated to contribute to the development of diabetic complications [9]. The mechanism of RAGE has also been widely discussed.

In this chapter, we will present data regarding the formation and the metabolism of AGEs, the role of RAGE involved in diabetic conditions, evidence emerging from in vitro and in vivo studies as well as studies using anti-AGEs and other related agents to support a pathogenic role for AGEs in the impaired process of diabetic wound healing.

2. AGEs formation

It was not until 1980 that the pathophysiological significance of AGEs emerged in medical science, particularly in relation to diabetic complications [10]. AGEs are a heterogeneous group of molecules that form from the non-enzymatic addition of sugar moieties onto arginine and lysine residues of proteins, free amino groups on lipids, or guanine nucleic acids [11]. Glycation has to be distinguished from glycosylation, which is an enzymatic reaction. First described by Louis Camille Maillard in the 1900s, non-enzymatic glycation involves condensation reaction of the carbonyl group of sugar aldehydes with the N-terminus or free-amino groups of proteins via a nucleophilic addition, resulting first in the rapid formation of a Schiff base. The physiological consequences of the Maillard reaction in the etiology of a range of important diabetic complications have already been indicated [12]. The Schiff base then goes through rearrange-

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ments to form the more stable Amadori products. Among most cellular and plasma proteins, Amadori products can change with glucose. That is to say, the levels of Amadori products will rise and fall depending on the levels of glucose. The most well-known example of an Amadori product is hemoglobin A1c (HbA1c), a naturally occurring modification to the N-terminal valine amino group of the β chain of hemoglobin [13]. Schiff bases and Amadori products are reversible reaction products. However, they can react irreversibly with amino acid residues of peptides or proteins to form protein adducts or protein crosslinks [14] (**Figure 1**).

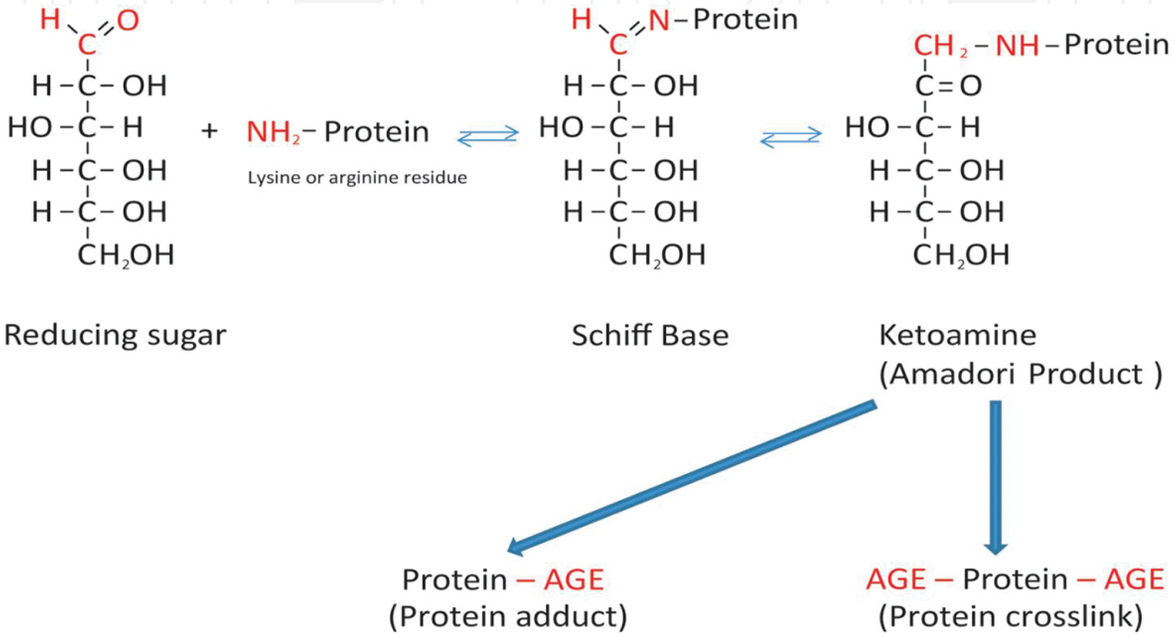

Figure 1. Schematic presentation of the Maillard reaction. Reactive carbonyl groups of a reducing sugar react with neutrophilic free amino groups of proteins to form a reversible Schiff base. Through rearrangement, a more stable Amadori product is formed. Depending on the nature of these early glycation end products, protein adducts or protein crosslinks are formed. (Illustrated from Ref. [54]).

In the context of intracellular glycation, it is important to note that glucose has the slowest rate in the glycation reaction of any sugar [15]. Because of the slow formation, it is believed that AGEs accumulate only on long-lived extracellular proteins. However, later a rapid extracellular AGEs formation on short-lived proteins and intracellular AGEs formation by reactive dicarbonyl compounds have attracted attention [16]. Thus, glycolytic intermediates such as dihydroxyacetone-phosphate, glyceraldehyde-3-phosphate and the dicarbonyl compounds glyoxal, methylglyoxal, and 3-deoxyglucosone are important for the intracellular Maillard reaction [17]. Among these compounds, methylglyoxal is regarded as the most potent glycating agent [18]. The transformation of glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate formed methylglyoxal [19]. It could be detoxified by the conversion to S-Dlactoylglutathione and D-lactate, catalyzed in the cytosol of all cells by glyoxalase I and II. It has been reported that overexpression of glyoxalase I in endothelial cells completely prevented AGEs formation, thus indicating the importance of methylglyoxal to form AGEs [20]. Moreover, several studies on different animal models have established that dietary AGEs could play

an important role in the pathogenesis of various pathologic conditions and their complications, such as type 1 diabetes mellitus in non-obese diabetic mice [21], atherosclerosis in apoE-deficient mice [22], type 2 diabetes, and impaired wound healing in db/db (+/+) mice [23]. It should be emphasized that a large portion of AGEs in the human body is derived from exogenous sources, e.g. from regular food, smoking, etc. [24]. Much attention has been paid to the so-called exogenous AGEs, harmful products of "browning" (or the Maillard reaction) in various foods. Together with endogenous AGEs, these compounds form the majority of glycation-free adducts. Among the various food processing methods, heating, sterilizing, and microwaves contribute to the generation of exogenous AGEs, all of which tend to accelerate the non-enzymatic addition of non-reducing sugars to free NH2 groups of proteins and lipids [25].

3. RAGE

AGEs could exert their actions not only directly but also through a receptor system, which includes two types of cell surface AGEs receptors: first type is that binds AGEs and initiates cell activation and second type is that binds and degrades AGEs. Receptor for AGEs (RAGE) is one receptor of the first type; it recognizes AGEs and initiates oxidative stress. The second type of receptors consists of AGER1, AGER3, and CD36 [26, 27]. However, it is noteworthy that there are other AGE receptors, such as the macrophage scavenger receptor and the galectin-3 receptor, which might have similar deleterious effects to RAGE when they interact with AGEs [28].

RAGE is a multi-ligand receptor of the immunoglobulin superfamily of cell surface molecules acting as a receptor not only for several molecules including AGEs but also for S100/calgranulins and amyloid. Circulating isoforms of RAGE include soluble RAGE (sRAGE) that has been cleaved from the cell surface by matrix metalloproteinases and endogenous secretory RAGE (esRAGE), and a splice variant of RAGE that is secreted into blood. Both sRAGE and esRAGE protect body against the AGEs-elicited tissue damage by acting as a decoy receptor for AGEs [29, 30]. The ligands of RAGE have a common feature that they accumulate in tissues during aging, inflammation, and degenerative diseases. Engagement of RAGE results in intracellular signaling that leads to the activation of NF-kB, a pro-inflammatory transcription factor, which is then translocated to the nucleus and subsequently activates the transcription of target genes [31]. These include genes of cytokines, adhesion molecules, and prothrombotic and vasoconstrictive products. The activation of NF-kB results in upregulation of the receptors in return. In addition, cellular-signaling cascades such as the ERK signaling pathway and PI-3 kinases are activated by the binding of ligands with RAGE [32].

In the skin, RAGE expression was observed in both epidermis and dermis, and it was increased in sun-exposed compared with UV irradiation-protected areas [33]. Not only in vivo, but also in vitro, various skin cells types have been shown to express RAGE [34–36], such as keratinocytes, fibroblasts, dendritic cells, and to a lesser extent endothelial cells and lymphocytes. Patients with diabetes also exhibit increased immunoreactivity for RAGE and AGEs. For

example, in sural nerve biopsies, AGE-RAGE interaction was found which suggests it may have a clinical role in neuronal dysfunction that leads to neuropathy [37].

According to these reactions, researchers have put forward mechanisms by which AGEs lead to diabetic complications: (1) the accumulation of AGEs in the extracellular matrix causing aberrant crosslinking, resulting in a decrease of elasticity of vessels; (2) intracellular AGEs formation leading to quenching of nitric oxide and impaired function of growth factors [20]; (3) the binding of AGEs to AGE-receptors on different cell types and activation of key cell signaling pathways such as NF-kB activation with subsequent modulation of gene expression in vascular cells such as endothelial cells, smooth muscle cells, and macrophages [38].

4. AGEs and wound healing

It is generally believed that wound healing is impaired in diabetes. Wound healing is a complex process in which several pathophysiological processes are involved. They include inflammation, repair, and regeneration. Until now, there is evidence from experimental studies that glycation is involved in wound healing in diabetes.

4.1. AGEs and inflammation phase in a diabetic wound

In this part, the interaction between AGEs and RAGE could not be neglected. Data support that it negatively affects various aspects of inflammatory response in diabetic wound. Increased RAGE expression has been found in wound tissues from diabetic mice in parallel with increased AGEs accumulation and increased inflammation [39]. In Shuliang Lu's study [40], compared with the controls, enhanced expression of RAGE and accelerated cell apoptosis were observed in the burned skin of diabetic rats. The altered expression pattern of inflammatory cytokines and oxidative markers between diabetic and control groups revealed delayed neutrophil chemotaxis and respiratory burst. Furthermore, the results in vitro showed that exposure to AGEs inhibited the viability of neutrophils, promoted RAGE production and cell apoptosis, which was consistent with the findings in vivo. Besides, the mice fed with a rich AGEs diet demonstrated an increased and sustained inflammatory phase compared with those fed with a low AGEs diet [41]. In vitro, human neutrophils were isolated and treated with AGE-human serum albumin. Cell viability and reactive oxygen species levels were increased [42].

In keratinocytes, AGEs decrease cell viability and migration and induce the expression of proinflammatory mediators as well [43]. Various growth factors or proteins significant for cellular functions may be glycated inhibiting their functions [44]. Furthermore, treatment of murine macrophages with AGEs resulted in increased levels of iNOS, which has been found to be increased in diabetic wounds [45]. Macrophages play a critical role in wound healing and can be activated to two distinctive phenotypes in vitro: M1 and M2 [46]. It demonstrated insufficient M1 in the early stage but excessive M2 in the later proliferative phase. The macrophage activation markers were correlated with the instructive T helper cell type 1 (Th1)/

Th2 cytokines in both groups. Other studies suggested that RAGE expression has been strongly linked to the expression of matrix metalloproteinases (MMP)-1, MMP-3, MMP-9, mainly through RAGE engagement by AGEs [47]. In addition, AGEs induced the production of oxygen-reactive intermediates from inflammatory and endothelial cells via NADPH activation probably through their receptors, promoting further cellular activation and proinflammatory cytokine expression [48].

4.2. AGEs and proliferation phase in the diabetic wound

It has been reported that the presence of AGEs not only affected the interaction of the fibroblasts with the extracellular matrix but also reduced the amount of the extracellular matrix as well. This effect would influence almost all the cells involved in the proliferative process. In vitro incubation of human dermal fibroblasts with pentosidine or pyrraline resulted in reduction of the extracellular matrix content, which was collagen and proteoglycan [49]. In vitro study showed that type I collagen synthesis from fibroblasts was not affected in AGEs; however, the synthesis of hyaluronic acid was significantly reduced [50]. It also showed a direct effect of AGEs on fibroblast synthetic capacity and explained the decreased extracellular matrix in the diabetic wound. Because hyaluronic acid is associated with cellular locomotion, migration, and proliferation, decreased content in the matrix could result in disturbance of the proliferative phase of the healing process. Besides, histological evaluation of wound sections from diabetic rats demonstrated absence of actively migrating inflammatory cells toward the central region of the wound, reduced angiogenesis, a decrease in the secretion of extracellular matrix, and then poor granulation tissue formation [51].

AGEs may also change the action of the wound-associated cytokines and growth factors, by affecting the growth factors or their receptors. Glycation of bFGF, after its incubation with glucose-6-phosphate (G6P) or fructose, resulted in decreased heparin-binding capacity, which is necessary for the binding of bFGF to its receptor. A reduction in its mitogenic activity was also observed compared with the control bFGF group [45]. In addition, incubation of FGF2 with G6P resulted in glycation of FGF2. Bovine aortic endothelial cells incubated with the glycated FGF2 showed a reduction of proliferation, decreased mean capillary length and new blood vessel formation, a weaker increase in tyrosine-phosphorylated proteins, especially ERK-1 and ERK-2 [52] .While ECV304 cells were incubated with glyoxal proteins, a significant reduction in the free amino acid groups of the EGF receptors was found. It also showed that the EGF-induced recruitment and activation of the downstream effectors of the EGF receptor pathway PLCg1 and ERK1/2 was inhibited by AGEs [53]. Furthermore, an animal study using rats with subcutaneous implantation of sponge disks showed that pretreatment of animals with D-glucose resulted in a reduction in the angiogenesis measured by the hemoglobin content of the implanted disks and a reduction in the granulomatous response compared with control groups.

4.3. AGEs and remodeling phase in the diabetic wound

Every stage of normal wound healing appears to be disrupted in the diabetic patients. A derangement in wound contraction and remodeling is expectable. A large body of evidence

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supports the effects of AGEs on the phenotype, invasiveness, behavior, and survival of the cells and cell membrane interactions with extracellular matrix. Animal data showed that diabetic mice with lower circulating and tissue-bound AGEs as a result of exposure to a diet low in AGEs, showed improved reepithelialization, granulation tissue formation and angiogenesis compared with the group fed with a diet high in AGEs [54].

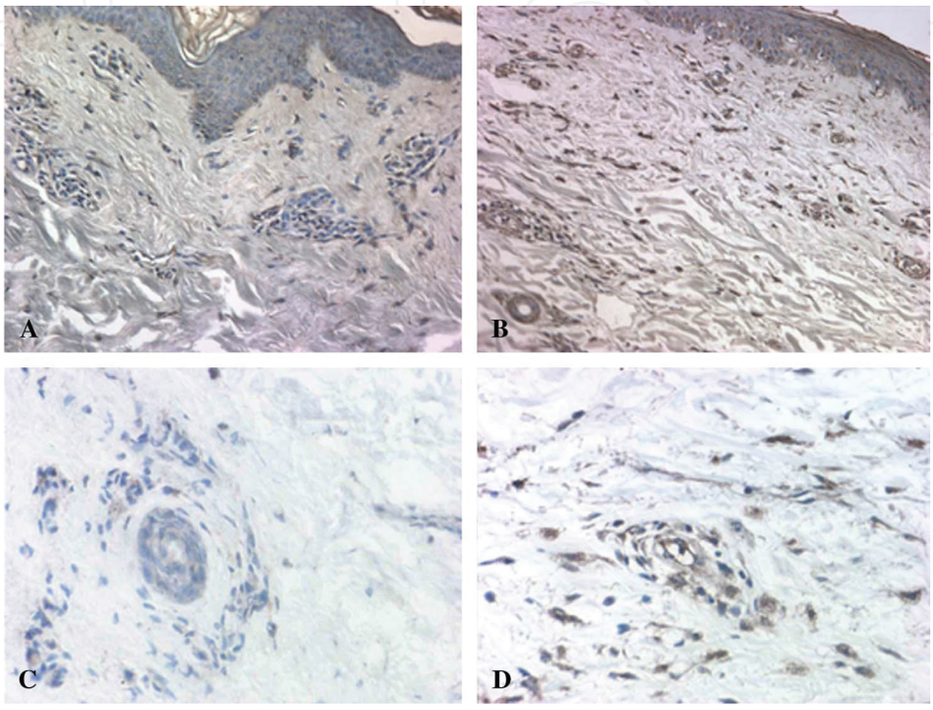

Figure 2. Immunohistochemical localization of AGE and RAGE proteins in dermis is shown. A, B, The distribution of AGE in normal skin tissue (A) and in diabetic skin tissue (B). AGE protein staining was expressed faintly at dermal matrices and cells in control skin but was prominent at the dermal matrices, cells, and basement membrane of vessels in the diabetic skin. C, D, The distribution of RAGE in normal skin tissue (C) and in diabetic skin tissue (D). RAGE-positive cells appear brown, and a light hematoxylin counter stain was used to visualize nuclei. More positive cells were detected in diabetic dermal layer than in control. [Original magnification, ×200 (A, B); original magnification, ×400 (C, D)]. (Illustrated from Ref. [55]).

The balance between proliferation and apoptosis of skin cells is responsible for the success of the wound healing process. Recent reports have shown that AGEs formation participates in dermatologic problems in diabetes. Shuliang Lu's group reported that effects of dermal micro-environment glycosylation. Histology and immunohistochemical staining were performed on type 2 diabetic and nondiabetic skin specimens to determine the distributions of proliferating cell nuclear antigen, apoptotic cells, AGEs and RAGE. Diabetic skin has degenerative, loosely arranged collagen and increased apoptotic cells compared with normal skin. Expression of AGEs and RAGE were increased in diabetic skin. Glycosylated matrix induced cell cycle arrest and apoptosis of cultured dermal fibroblasts, whereas application of RAGE-blocking antibodies redressed these changes [55] (**Figure 2**).

AGEs may alter the signaling of the wound cytokines and growth factors by disrupting the structure of either the growth factors or their receptors. Glycation of fibroblast growth factor

(bFGF), after its incubation with intracellular sugars resulted in decreased heparin-binding capacity, which is essential for the ligation of bFGF to its receptor [56]. Bovine aortic endothelial cells incubated with the glycated FGF-2 showed a reduction in the proliferation, decreased mean capillary length and overall new blood vessel formation as well as a clearly weaker increase in tyrosine phosphorylated proteins, particularly ERK-1 and ERK-2 [57]. It has also been shown that the epidermal growth factor (EGF)-induced recruitment and activation of the downstream effectors of the EGF receptor pathway, the serine-threonine kinases ERK1/2, was inhibited by glyoxal and methylglyoxal [58]. Moreover, diabetic rats exhibited poor TGF-β1 expression in fibroblasts [20]. The effects of TGF-β1 on extracellular matrix synthesis and cellular phenotypes are crucial for the final stage of wound healing, suppression of MMP secretion, differentiation of fibroblasts into contractile myofibroblasts, and cellular programmed death. The increased levels of MMPs and proinflammatory cytokines in the context of a vicious self-perpetuating cycle of an inappropriately inflammatory response may be responsible for the derangement of the remodeling stage [42].

Literature data also support that in the presence of AGEs, not only the interaction of the fibroblasts with the extracellular matrix is affected, but also the amount of the extracellular matrix constituents normally secreted by cells is reduced. This effect would deprive almost all the cells involved in the proliferative process of the extracellular scaffold [59]. In vitro, studies showed a direct effect of AGEs on fibroblast survival and synthetic capacity, which might partially explain the decreased extracellular matrix density in the diabetic non-healing wounds. Human adult primary skin fibroblasts treated with CML-collagen (glycated collagen) showed a time- and dose-dependent apoptosis, which was threefold compared with that of control collagen-treated fibroblasts [60]. An insight has been gained into the mechanisms that underlie the AGEs-promoted cell apoptosis. The proapoptotic intracellular signaling consists of involving a chain of events, such as the generation of intracellular reactive oxygen species, which cause the activation of mitogen-activated protein kinase (MAPK) pathways and finally the induction of transcription factor FOXO1 and caspase-3. In addition, keratinocytes pretreated with glycoaldehyde and type I collagen exhibited reduced migration and an impaired adhesive capacity [61]. These effects were caused by conformational changes on the glycated collagen, which altered the effective receptor binding [62]. Furthermore, increased AGE/RAGE expression has been found in the diabetic skin. The apoptotic effects could be reversed by the application of RAGE antibodies, suggesting that AGEs and RAGE interaction played an important part in the cell dysfunction [40].

Another study of Shuliang Lu's group demonstrated that thickness of abdominal dermis from diabetic patients was reduced with obscured multilayer epithelium and disorganized collagen fibrils, as well as with chronic inflammatory cell infiltration. It was also shown that the prominent accumulation of AGEs in the diabetic skin induced an oxidative damage of fibroblasts and thus contributed to the thinner thickness of diabetic abdominal dermis. In vivo, less hydroxyproline, higher myeloperoxidase activity, and increased malondialdehyde (MDA) content were found in the diabetic skin. In vitro, the time- and dose-dependent inhibitory effects of AGE-bovine serum albumin (BSA) on fibroblast viability and the promotion of MDA production were shown [63].

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5. Measurement of AGEs

Since the biochemistry of AGEs has been widely discussed, the effort to develop the measurement has been made as well. Blood is more accessible for repeated measurements of AGEs than tissue-requiring biopsies, but plasma AGEs assays have not yet been shown to be directly related to tissue AGEs content [64]. As tissue accumulation of AGEs proves a long-term course with low reversibility, the AGEs accumulated in long-lived tissue proteins like skin collagen may be a carrier of metabolic memory over a long period, even years [65]. Because certain AGEs have intrinsic fluorescence properties, tissue AGEs accumulation can be assessed as skin autofluorescence (SAF) by the AGE Reader™ (Diagn-Optics, Groningen, the Netherlands) easily and noninvasively [66], instead of other traditional invasive techniques (**Figure 3**). Several studies demonstrated that the SAF value obtained from the skin of the lower arm correlated with content of both fluorescent and nonfluorescent AGEs measured from skin biopsy specimens on the same site [67]. It is strongly related to AGEs accumulation in healthy subjects, and diabetic and hemodialysis patients over a broad age range [68]. There is no surprise that SAF values of the diabetic patients were significantly higher than the healthy population [69, 70], and SAF values of diabetes with complications were elevated compared with those without complications [71, 72]. So far, there have been SAF referential values and its influential factors for healthy Dutch, Slovakian, and Chinese people, and this offers the baseline values to further analyze diabetes and its chronic complications [73–75].

However, studies of SAF on predicting the diabetic vascular complications were of considerable clinical heterogeneity, and different experimental results were adjusted in different conditions [76, 77]. Recent publications have suggested that SAF serves as a marker of vascular damage [78], as well as a predictor of cardiac mortality in patients with type 1 and 2 diabetes. In Shuliang Lu's group, Liu Chuanbo et al. did a cross-sectional survey consisting of 118 consecutive hospitalized diabetic foot patients. The diabetic microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular referring to coronary heart disease (CHD), cerebrovascular disease (CVD), or peripheral artery disease (PAD) complications were evaluated .The mean SAF value was 2.8 ± 0.2 AU. SAF was significantly associated with diabetes duration and blood urea nitrogen ($R2 = 62.8\%$; $P < 0.01$). Moreover, in logistic regression analysis, SAF was significantly associated with retinopathy (odds ratio [OR] = 40.11), nephropathy (OR = 8.44), CHD (OR = 44.31), CVD (OR = 80.73), and PAD ($OR = 5.98 \times 109$). Therefore, SAF, reflecting tissue accumulation of AGEs is independently associated with the presence of micro-and macro-vascular complications in diabetic foot ulcer (DFU) patients [79]. Similarly, SAF values were significantly higher in type 1 diabetic patients with microvascular complications, like neuropathy, compared to those without complications [80]. Lisanne et al. reported that SAF was independently associated with all-cause mortality and fatal or non-fatal major adverse cardiovascular events in patients with peripheral artery disease after a 5-year follow-up [81].

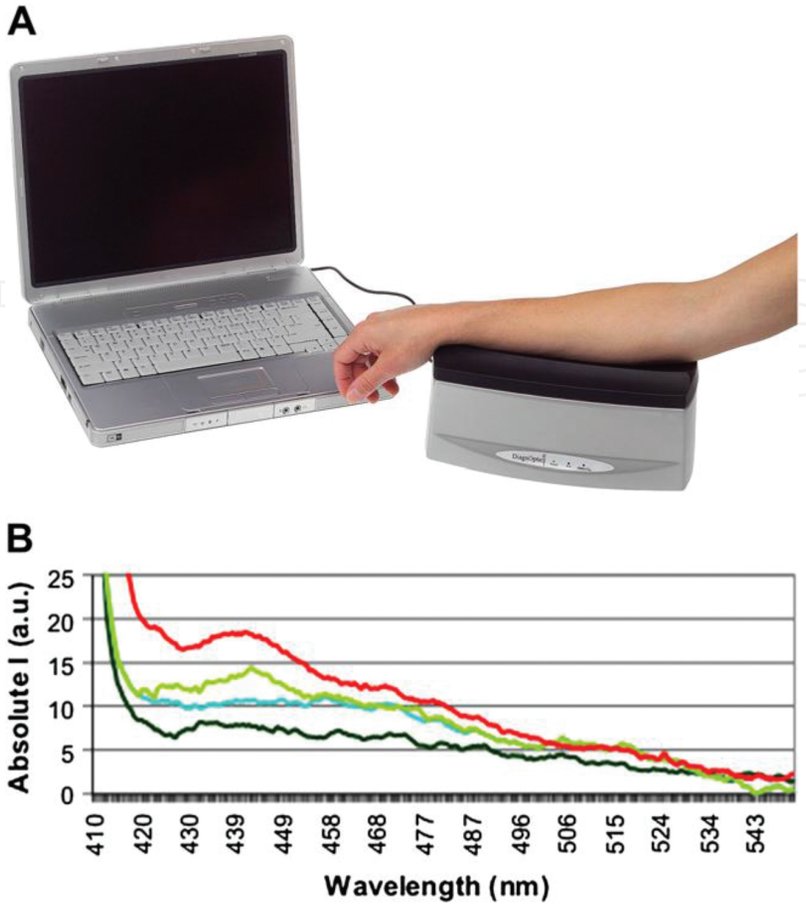

Figure 3. (A) The autofluorescence reader illuminates a skin surface with an excitation light source between 300–420 nm. Only light from the skin is measured with a spectrometer. (B) Various fluorescence spectrum results from different subjects: healthy subject (black line), diabetic patient without cardiovascular complications (blue line), diabetic patient with peripheral artery occlusive disease (green line), hemodialysis patient with recent myocardial infarction (red line). I = intensity (a.u.). (Illustrated from Meerwaldt R, van der Vaart MG, van Dam GM, et al. Clinical relevance of advanced glycation end products for vascular surgery. Eur J Vasc Endovasc Surg. 2008;36(2):125–31).

The use of SAF in the diabetic wound was also discussed. Meerwaldt et al. showed that SAF was increased and correlated with the Wagner score in DFU with neuropathy. SAF correlated inversely with nerve conduction velocity and amplitude [82]. Lapolla et al. found that AGEs were higher in type 2 diabetics with PAD compared to those without PAD; AGEs were correlated inversely to ABPI, even after correction for other cardiovascular risk factors [83]. SAF is independently associated with diabetic foot ulcerations. It might be a useful screening method for foot ulceration risk of diabetic patients [77].

Use of SAF measurement to assess foot vulnerability and to predict DFU events in high-risk patients seems to be promising. Yet, Vouillarmet et al.'s study in a subgroup of patients with an active DFU showed a nonsignificant correlation (P = 0.06) between SAF and the incidence of healing at 2 months, but the magnitude of effect is still high. Therefore, researchers deemed that the small number of patients may be the reason for the lack of statistical power. SAF method deserves attention because of its prognostic value for healing [84].

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However, long-term studies validating both the specificity and sensitivity of this investigation, and its link to certain AGEs, remain to be confirmed. The importance of its use in the follow-up of DFU is not reported. Thus, AGEs might have some value as a screening tool for DFU, but there is no strong evidence for other clinical use in diabetic wound, and AGEs measurements should not be considered a replacement for HbA1c as a marker of glycemic control.

6. Anti-AGEs strategies

Since AGEs were considered as an important factor in diabetes, the development of strategies against AGEs has been of interest. Substances, which can prevent or inhibit the formation of AGEs, as well as agents that can break AGEs or antagonize their signaling have been identified.

6.1. Inhibit the formation of AGEs

The first approach is to reduce the formation of AGEs by intervention at one of the steps involved such as aminoguanidine [85]. Aminoguanidine was one of the first substances identified limiting the formation of AGEs [86]. It is a highly reactive nucleophilic reagent that prevents the formation of AGEs by reacting with the carbonyl groups as well as alpha- and beta-dicarbonyl compounds such as methylglyoxal, glyoxal, and 3-deoxyglucosone. Particularly, long-term aminoguanidine treatment improved the nerve conduction deficit and myelinated fiber pathology in diabetic rats in vivo [87]. A double-blinded, multiple-dose, placebo-controlled, randomized clinical trial of aminoguanidine in diabetic patients with overt diabetic nephropathy (ACTION) was completed in 1998; ACTION I involved 690 type 1 diabetic patients and ACTION II involved 599 type 2 diabetic patients. These studies were designed to evaluate the safety and efficacy of aminoguanidine in slowing the rate of renal disease progression in patients with overt diabetic nephropathy. However, ACTION II was terminated prematurely due to safety concerns and apparent lack of efficacy. Reported side effects included gastrointestinal disturbance, liver function abnormalities, flu-like symptoms, and a rare vasculitis [88]. Its use in clinical practice is limited due to adverse effects in clinical trials with diabetic patients. Despite the earlier promising results, aminoguanidine is unlikely to be used for therapeutic purpose due to safety concerns and lack of efficacy [89]. Studies on topical application of aminoguanidine on the skin are still lacking.

Metformin that is routinely used in the treatment of type 2 diabetic patients has some structural similarities to aminoguanidine and it was shown that in type 2 diabetes, treatment with metformin reduced levels of methylglyoxal [90]. Pyridoxamine is a natural intermediate of vitamin B6 metabolism and a potent inhibitor of the formation of AGEs [91]. Pyridoxamine traps reactive carbonyl intermediates and scavenges ROS. In addition, it inhibits post-Amadori stages of AGEs formation. Marked effects of pyridoxamine such as delayed development of nephropathy and retinopathy have been demonstrated in diabetic rats. Its oral intake could result in potent inhibition of skin collagen CML formation in diabetic rats as well [92].

6.2. Anti-RAGE

RAGE is the most studied receptor for advanced glycation end products. AGER1 has been shown to counteract AGEs-induced oxidative stress via inhibition of RAGE signaling [93]. sRAGE is a truncated splice variant of RAGE containing the ligand-binding domain but not the transmembrane domain and has been found in plasma. sRAGE is a soluble extracellular protein without signaling properties and it is considered as a natural decoy receptor of RAGE [30].

Blockage of RAGE by sRAGE may be a new target for therapeutic intervention in diabetic disorders. Potential protective effects of sRAGE have been shown in various diabetes and inflammatory models [94]. Interestingly, sRAGE could also attenuate impaired wound healing in diabetic mice. Other promising effects in various systems have been shown in vitro and in vivo with neutralizing anti-RAGE antibodies [31]. Possible approaches include gene knock-down of RAGE by siRNA or anti-sense and antagonism of RAGE with putative small molecular inhibitors against RAGE-induced signaling [95].

6.3. AGEs breakers

Chemical substances and enzymes that are able to recognize and break the Maillard reaction crosslinks have been identified. Such chemical AGEs breakers are dimethyl-3-phenayl-thiazolium chloride (ALT-711) [64], N-phenacylthiazolium and N-phenacyl-4,5-dimethylthiazolium. Promising results against diabetic cardiovascular complications have been reported, though their actual ability to cleave existing protein crosslinks in tissues has been questioned [96]. However, treatment with ALT-711 for 2 weeks had no effects on motor nerve conduction deficit, C-fiber-mediated nociceptive dysfunction, or impaired pressure-induced vasodilation in diabetic mice [97].

Interference with intrinsic AGE-detoxifying enzymes like fructosyl-amine oxidases (FAOXs), fructosamine-3-kinase (FN3K), and the enzymatic system of glyoxalase I is another interesting strategy to remove AGEs, because enzymes could recognize specific substrates [60]. It is reported that overexpression of glyoxalase I significantly inhibits hyperglycemia-induced intracellular formation of AGEs in bovine aortic endothelial cells and in mouse mesangial cells by reduction of intracellular oxidative stress and apoptosis [98]. The pharmacological induction of such enzymes could represent a novel future strategy against AGEs.

Other anti-AGE agents, including the thiazolidine derivative named OPB-9195, have been investigated [99]. OPB-9195 has been shown to prevent the progression of diabetic nephropathy in rats. It has also been demonstrated to improve motor nerve conduction slowing without affecting body weight and blood glucose levels. The improvement was associated with reduced serum AGEs levels and peripheral nerve expression of AGEs and immunoreactive 8-hydroxy-2-deoxyguanosine, which is a marker for oxidative stress-related DNA damage as well as an increase in peripheral nerve (Na+, K+)-ATPase activity [100].

Diabetic rats were found to have increased mesenteric vascular AGEs accumulation and mesenteric vascular hypertrophy, both of which were prevented by treatment with N-

phenacylthiazolium bromide (PTB) [101]. A more recent study has demonstrated that although AGE-breakers such as PTB and N-phenacyl-4,5-dimethylthiazolium cleave model crosslinks in vitro, they do not significantly cleave AGE crosslinks formed in vivo in skin collagen of diabetic rats [59].

Benfotiamine, a lipophilic analogue of thiamine, is a transketolase activator that inhibits three of the four major biochemical pathways implicated in the pathogenesis of hyperglycemia-induced vascular damage: the hexosamine pathway, PKC activation, and AGEs formation [102]. In diabetic rats, nearly normalized nerve conduction velocity and inhibition of neural imidazole-type AGEs and CML formation after 6 months of benfotiamine treatment were observed [103]. In both nondiabetic and diabetic rats, benfotiamine also reduced inflammatory and neuropathic nociception [104].

6.4. Nutrient substance

An increasing list of natural antioxidants and chelating agents such as ascorbic acid, α-tocopherol, niacinamide, pyridoxal, sodium selenite, selenium yeast, trolox, rivoflavin, zinc, and manganese has been shown to inhibit glycation of albumin in vitro [105]. Many spices and herbs could inhibit glycation of albumin in vitro as well, such as ginger, cinnamon, cloves, rosemary, and tarragon [106]. Besides, green tea, vitamins C and E, and a combination of N-acetylcystein with taurine and oxerutin could inhibit skin collagen glycation in mice [107]. In healthy human subjects, supplementation of vitamin C significantly decreased serum protein glycation [108].

Alpha-lipoic acid could reverse tail tendon collagen glycation in fructose-fed rats, an effect which was attributed to its endogenous antioxidant action, its ability to recycle ascorbic acid and GSH, as well as to its positive influence on glucose uptake and glycemia [109]. Blueberry extract, an AGE-inhibitor and C-xyloside, was tested for 12 weeks in female diabetic subjects. This treatment resulted in significant improvement of skin firmness, wrinkles, and hydration. However, it failed to show a significant decrease in the cutaneous content of AGEs [110].

6.5. Molecular chaperones

Molecular chaperones like carnosine have shown promise in improving skin appearance in part by reducing the amounts of skin AGEs [111]. Yet, more studies are needed to address the accumulation of AGEs in diabetic wound.

In conclusion, AGEs are a heterogeneous group of molecules that form from the non-enzymatic addition of sugar moieties onto arginine and lysine residues of proteins, free amino groups on lipids, or guanine nucleic acids. The AGE-RAGE interactions play an important role in the diabetic wound healing process. The measurement of AGEs on the skin, namely, skin autofluorescence might have some value as a screening tool for diabetic foot ulcer, but until now, there is no strong evidence for other clinical use in diabetic wound. In addition, substances which can prevent or inhibit the formation of AGEs, as well as agents that can break AGEs or antagonize AGE/RAGE signaling have been identified.

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