Introduction

Reducing sugars, such as glucose in basic solutions, and lipids by β-oxidation or peroxidation generate formyl (aldehyde) and ketone groups. Aldehydes and ketones have a highly polarized carbonyl (C=O) group, the oxygen atom of which is electronegative and may react with nucleophiles in proteins. As a result, aldehydes and ketone groups may react non-enzymatically with cell proteins by glycation, and degrade protein function. Although similar reactions have been observed in vivo, we have little information on the relationship between these reactions and age-related changes in skin.

Many age-related regressive changes are actually due to protein degradation, such as posttranslational modification (proteomic denaturation), accumulation of degenerated wastes, deterioration of functional proteins, functional disorder of the tricarboxylic acid (TCA) cycle, or activation of inflammatory pathways by intracellular signals. All of these changes are symptomatic of "glycation stress".

Glycation stress includes a number of different chemical reactions. For example, a Maillard reaction is defined as a non-enzymatic/irreversible reaction of reducing sugar and protein. This reaction may generate advanced glycation end products (AGEs) by a chain of various intermediate reactions that produce Schiff bases and Amadori products. A Schiff base (general structure R1R2C=N-R3, where R3 = alkyl or aryl or similar group, but not H) is a reversible compound generated from the interaction of a reducing sugar, such as glucose, and lysine or arginine amino acids or the N-terminal amino group in a protein. A Schiff base may be further modified by Amadori rearrangement. For example, hemoglobin A1c is an irreversible ketoamine formed by glycation of hemoglobin. The overall reaction is divided into three parts, an initial reaction, intermediate product generation, and an advanced reaction (Fig. 1).

Intermediate products include 3-deoxyglucosone (3DG), glyoxsal, methylglyoxal (MG), glycolaldehyde (GA), glyceraldehyde, are also carbonyl compounds that may generate AGEs. For example, 3DG is an α-carbonyl compound 10,000 times as reactive as glucose generated...
Glycation Stress and Photo-Aging in Skin

from an Amadori product. When plasma 3DG concentration rises above 100 nmol/l, the risk of diabetic retinopathy and nephropathy doubles. Several other AGEs have also been implicated in the pathology of age-related diseases.

In other reactions, excess levels of reducing sugars, such as intracellular glucose, may affect mitochondria and disturb the TCA cycle by inducing production of excess fumaric acid. In turn, fumaric acid reacts rapidly with the amino acid cystein in proteins, finally forming S-(2-succinyl)cysteine (2SC). The resulting protein modifications provoke functional disorders in vivo. Proteins that are susceptible to this reaction include cytoskeleton protein, heat shock protein, and adiponectin.

Once AGEs are generated, they are metabolized by protease and oxidized protein degradation enzymes, such as oxidized protein hydrolase (OPH), in the proteasome and then excreted. OPH is a serine protease widely distributed in vivo that preferentially degrades oxidized or glycated protein by proteolysis. OPH is known to be an acylamino-acid-releasing enzyme (AARE) which separates acylated amino acids from protein at the N-terminal. Although the precise functions and activation of AAREs are unclear, OPH may modify protein carbonylation. OPH serum levels are higher in diabetic rats than in non-diabetic rats, and the amount of protein modified by carbonylation is lower in serum with elevated OPH activity than in relatively low-activity samples.

Other enzymes are also known to modify AGEs and intermediate compounds. For example, glyoxalase 1 (GLO1) metabolizes methylglyoxal (MG), an intermediate product of glycation stress. In experimental animals, renal damage induced by ischemia and reperfusion was inhibited by gene over-expression of GLO1. Although OPH and GLO1 are integral components of the excretion pathway for AGEs and intermediate compounds, the activity of proteolytic enzymes decreases with age. For this reason, therapies that reduce AGE accumulation by activating the proteasome, OPH, or GLO1 may prove effective in treating age-related disease or other diseases linked to glycation, such as diabetes mellitus.

Glycation stress also includes secondary or derivative reactions (Fig. 1), and these reactions can be implicated in three major diabetic complications: neuropathy, nephropathy, and retinopathy. These diseases are characterized by a marked accumulation of AGEs in tissue. Glycation stress is extremely high in hyperglycemia patients, where plasma glucose concentration exceeds 160 mg/dl, and may also be elevated in response to aldehydes generated by excessive alcohol consumption, hyper-triglyceridemia, or hyper-low-density lipoprotein (LDL)-cholesterolemia.

**Glycation stress markers**

Glycation generates various substances that can be used to identify glycation stress. For example, glycated hemoglobin (HbA1c) and glycoalbumin are Amadori products generated via Schiff base formation and are used as markers for diabetic evaluation. Other AGEs that may be used as glycation stress markers, including \( N^\epsilon -(\text{carboxymethyl}) \text{ lysine (CML)} \), pentosidine, pyrraline, crossoiline, \( N^\omega -(\text{carboxyethyl}) \text{ arginine (CEL)} \), and \( 2-(2\text{-furoyl})-4(5)-(2\text{-furanyl})-1H\text{-imidazole} \).

CML is a non-fluorescent and non-cross-linked AGE generated from intermediate glyoxsal and can be observed in patients with diabetes or high oxidative stress. CML may react with collagen, and CML-ized collagen added to a culture of human dermal fibroblast induces apoptosis. CML is present in the skin, especially epidermal layer where metabolic turnover is higher than in deeper layers. CML attaches to fluorescent anti-CML polyclonal rabbit antibody and can be observed on fluorescent microscopy (Fig. 2).

Pentosidine is another common AGE, fluorescent and cross-linked and generated from ribose, arginine, and lysine. This compound is recognized as an early clinical marker for nephropathy. Recently, pentosidine has been used as a marker for detecting osteoporosis (bone aging). Pentosidine is also found in dermal collagen, and concentrations increase with age. After adjusting for age, the accumulated concentration of pentosidine is much higher in diabetic patients than in healthy persons.

**Fig. 1.** Concept of glycation stress.

AGEs: advanced glycation end products, RAGE: receptor for AGEs, TCA: tri-carboxylic acid.
Receptors for AGE

In addition to disrupting cell function by protein modification, AGEs may also combine with a specific receptor for AGEs (RAGE). RAGE may play a pathogenetic role as AGE receptors by modifying intracellular signals and responses, such as activating cell signals that generate inflammatory cytokines 1. Many other cell surface receptors are reported to recognize AGEs as a ligand besides RAGE. One such intracellular signaling pathway amplifies oxidation stress and activates the transcription factor NF-κB, which is mediated by the ras/MAP kinase pathway. In vascular endothelial cells, AGEs stimulate a RAGE-induced signal that executes gene expression of vascular endothelial growth factor (VEGF). VEGF augments vascular permeability and angiogenesis and generates vascular cell adhesion molecule-1 (VCAM-1), which enhances local inflammation 27.

Although RAGE is usually bound to cell membranes, some may be soluble outside cells (soluble RAGE) 1. Soluble RAGE may bind with AGEs, acting as a decoy receptor and thereby inhibiting activation of RAGE on cell membranes 27. Presence of soluble RAGE may therefore indicate resistance to glycation stress.

Glycation stress in skin

Accumulation of AGEs such as CML in dermal tissue greatly influences glycation stress in the skin. Recent reports have shown that carboxylated protein deposited in the outer layer of epidermis changes the optical characteristics of dermal cells by reducing skin transparency 20. AGEs formed in the corium cause skin yellowing 29. In the corneum, where cell turnover is rapid, K10 protein generated by differentiation of keratinocytes can be susceptible to AGE formation 30.

AGEs accumulate in skin partially by binding with collagen protein. A collagen fiber forms a triple-helical structure in vivo and maintains skin elasticity in combination with elastin fiber. Lysine and arginine amino acids in collagen may be glycated to AGEs and form cross-links between fibers, thus reducing elasticity 31 (Fig. 3). Glycation of collagen may also cause loss of skin elasticity and wrinkle formation, although this has yet to be confirmed.

A second example of AGE-induced glycation stress arises from pentosidine. This compound is a strong activator of NF-κB, and induces cytokines generation and causes inflammatory skin changes.

Photo-aging and AGEs

Symptoms of photo-aging, such as spots (lentigo seniles), wrinkles, or tumors, frequently develop in skin repeatedly exposed over many years to ultraviolet (UV)-A, UV-B, or infrared light from the sun. After age 40, small spots may develop on the back of the hands, and the number of spots may increase with age 32. These spots also develop in patients with xeroderma pigmentosum, a rare hereditary skin disorder caused by a defect in the enzymes that repair DNA damaged by ultraviolet light, a few months after birth. These previous findings suggest that this spot formation may result from mutation of a gene involved in melanin formation in epidermal keratinocytes and melanocytes 33. UV-A exposure-induced mutation of a gene controlling a transcription factor, such as stem cell factor (SCF), might alter gene expression in keratinocytes. One possible SCF receptor found in melanocytes, c-kit, is implicated in spot development, but the precise mechanism is unknown.

Fig. 2. Distribution of CML in human skin. Fluorescent immunohistochemistry using anti-CML polyclonal rabbit antibody 23. Green fluorescence indicates presence of CML in the epidermal layer. CML: Nε-(carboxyl)methyllysine. Cross-section of human skin. Bar indicates 20 µm.
A further cause of changes in skin color and transparency may be delayed turnover in the epidermis. UV-A light may delay epidermal turnover by inactivating enzymes that promote keratinocyte detachment and peeling of the horny layer, although this mechanism is also unclear. All of these alterations are induced by photo-aging and intensified by AGEs accumulation\(^{22,23}\).

**Reduction of skin elasticity**

The reduction in skin elasticity in humans with age (Fig. 4)\(^ {34}\) is assumed to be caused by a reduction in elastic fiber production, i.e. collagen, elastin, and extracellular matrix ingredients such as fibronectin, possibly due to some disorder in fibroblast function. However, collagen protein also deteriorates due to oxidation or glycation. The skin elasticity curve is shifted downwards in patients with type 2 diabetes compared with that of healthy subjects, implying that glycation stress is a major factor in the reduction of skin elasticity.

Wrinkles appear on the face after age 30 as proteins, elastic fibers and collagen generated by fibroblasts in the dermis change, mainly due to solar irradiation\(^ {35-37}\). Low-dose solar irradiation elevates metalloproteinase concentration, by activating AP-1 and NF-κB which degrade collagen and elastin\(^ {35}\), and reduces the production of procollagen type I\(^ {36}\). Shallow wrinkles may be caused by structural changes, such as skin dryness; deeper wrinkles form on the face and neck of persons chronically exposed to sunlight, such as agricultural workers. In some of these workers, deep, triangle-shaped wrinkles called “diamond-shaped skin” appear on the neck by age 50, and the skin may appear yellowish and feel like hard cloth. In diamond-shaped skin, anti-CML antibody-positive substances are deposited in clumps in the middle and upper layers of the dermis, a condition known as solar elastosis. The clumps are detectable by staining elastin via van Gieson’s method\(^ {23}\).
characteristic wrinkles are not seen in facial skin before the age of twenty or in older persons not exposed to UV light.

UV-B light also stimulates production and release of inflammatory cytokines, such as IL-1α, IL-6, and TNFα from keratinocytes in the epidermis. In turn, the cytokines stimulate fibroblasts in the dermis and keratinocytes through the autocrine and paracrine systems and elevate mRNA formation and synthesis of matrix metalloproteinase (MMP)-1, MMP-3, or MMP-9, enzymes which degrade collagen and elastic fibers. MMP-1 is known to cut fibrous protein and may be responsible for wrinkle formation. Elastase is another enzyme which breaks down elastin, and inhibition of elastase has been found to reduce UV exposure-induced skin wrinkle formation in experimental animals. Taken together, these previous findings indicate that wrinkle formation is due to quantitative and qualitative changes in elastic fibers, which may be stimulated by UV light.

UV-A may also act directly on fibroblasts in the corium, enhancing mRNA formation and protein enzyme synthesis. Reactive oxygen species (ROS) induced by UV-A, UV-B, and infra-red light may also elevate levels of MMP-1 mRNA, in turn leading to further collagen degradation. Given that mitogen-activated protein kinases (MAPK) have been implicated in this intracellular signal pathway, anti-oxidants may effectively prevent wrinkle formation.

**Non-invasive skin AGEs examination (AGE Reader)**

The AGE reader™ (DiagnOptics, Groningen, Netherlands) is designed to non-invasively evaluate the level of AGEs accumulated in skin, utilizing the principle that accumulated AGEs emit characteristic autofluorescence (AF) when excited by UV light (Fig. 3). The AF of immuno-histochemical skin biopsies from patients with diabetes mellitus and nephropathy receiving dialysis is correlated with the level of accumulated AGEs, such as pentosidine or CML, although CML is a non-fluorescent AGE.

Measurements of skin AF in healthy Japanese females show that AF intensity increases with age (Fig. 5). In Europe and the USA, it is common practice to measure the skin AF for Caucasian patients from the upper forearm. However, results from the present authors’ previous research showed that precise measurement of AF in suntanned Asian subjects was difficult. Clinical practice in Japan is to have the subject rest his or her cheek on his or her hands and measure the AF from a 10-cm length of skin, on the back of the arm above the elbow. Further data will be required to validate this method of measuring skin AF for Asian patients.

Precisely which part of the skin fluoresces in response to the AGE reader or the types of AGE detected and their locations within the dermis remain unclear at present using this method. We assume that the AGEs measured by the AGE Reader are pentosidine, crossline, and pyrropiridine.

**Prevention and treatment of glycation stress**

To prevent skin aging, potential treatments must be effective against photo-aging, oxidative stress, and glycation stress. Primary prevention is achieved by encouraging maintenance of reasonable skeletal muscle mass, moderate exercise, and proper eating habits. These recommendations will help ensure even concentrations of blood sugar and reduce insulin resistance.

Muscle load training is particularly important. More than 70% of blood glucose is consumed in skeletal muscle, and reduction of muscle mass increases insulin resistance. Eating habits that reduce glycation stress are to eat slowly, chew food well, choose foods that do not raise blood sugar rapidly (i.e., foods with a low glycemic index), and avoid high sugar content foods, such as juice, carbonated drinks, and sweets. We also recommend moderate or minimal alcohol intake, as alcohol metabolites enhance glycation stress in vivo.

Persons with excessive weight or waist size should...
receive medical checkups and manage their blood sugar, LDL-cholesterol, and triglyceride levels. Numerical targets for each measure should follow the guidelines published by the Japan Diabetes Society or Japan Society of Ningen Dock.

Further treatment with anti-glycation materials may be provided if necessary after lifestyle factors have been corrected. In the future, we expect that the following therapies may be provided to treat AGE-related disorders: AGE generation inhibitors, AGE breaker that enhance AGE metabolism \(^{46,47}\), and AGE receptor antagonists \(^{48}\). Aminoguanidine is one such AGE generation inhibitor \(^{49,50}\) but is associated with a high frequency of side effects and is not used clinically in Japan. A mixture of herbal extracts from chamomile (\textit{Anthemis nobilis}), hawthorn berry (\textit{Crataegus oxyacantha}), dokudami (\textit{Houttuynia cordata}), and grape leaf (\textit{Vitis vinifera}) has been found effective in inhibiting AGE formation \(^{48}\), in experimental animals \(^{51}\), and in clinical trials \(^{49,52}\). Astragaloiside, which is derived from \textit{Astragalus radix}, is reported to significantly inhibit generation of CML and pentosidine \(^{53}\). AGE decomposers include thiazolium compounds such as N-phenacylthiazolium and N-phenacyl-4,5-dimethylthiazolium \(^{46,47}\).

Medicines may eventually be developed which target RAGE and related compounds. Full-length membrane-bound RAGE helps induce intracellular signal transmission and formation of inflammatory cytokines. In contrast, soluble RAGE (sRAGE) acts as decoy receptors that competitively bind to AGEs and assist in AGE clearance, thereby inhibiting RAGE-induced signal transmission. sRAGE is shed from the ectodomain of flmRAGE and activated by MMP 9 or a disintegrin and metalloproteinase (ADAM) \(^{10}\). Therefore, treatments that reinforce ectodomain shedding will decrease the total levels of flmRAGE and in turn increase the levels of sRAGE, thereby modifying AGES-RAGE signaling and subsequent cellular and tissue damage. Further clarification of this mechanism will be required to develop new methods of controlling RAGE ectodomain shedding.

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