Glycated lysine residues: A marker for non-enzymatic protein glycation in age-related diseases

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Abstract. Nonenzymatic glycosylation or glycation of macromolecules, especially proteins leading to their oxidation, play an important role in diseases. Glycation of proteins primarily results in the formation of an early stage and stable Amadori-lysine product which undergo further irreversible chemical reactions to form advanced glycation endproducts (AGEs). This review focuses these products in lysine rich proteins such as collagen and human serum albumin for their role in aging and age-related diseases. Antigenic characteristics of glycated lysine residues in proteins together with the presence of serum autoantibodies to the glycated lysine products and lysine-rich proteins in diabetes and arthritis patients indicates that these modified lysine residues may be a novel biomarker for protein glycation in aging and age-related diseases.

Keywords: Lysine, Glycation, Amadori products, AGEs, autoantibodies, diabetes, biomarker

1. Introduction

A non-enzymatic glycosylation or Maillard reaction of proteins primarily takes place at ε-amino groups of lysine or their free amino groups. The side chains of arginine, histidine, tryptophan and cysteine residues are the other sites for glycation [48]. Amadori-modified proteins, an early glycation product, undergo further reactions through a number of pathways and giving rise to advanced glycation end products (AGEs) [73,76]. Reactive carbonyl species (RCS), such as glyoxal, formed from antioxidative degradation of glucose and from other metabolic activities, react with lysine residues of proteins to form Nε-carboxymethyllysine (CML), a non-fluorescent and non-cross linking AGE [15]. CML is also formed by oxidative degradation of Amadori products [50]. A scheme for the formation of Amadori products and AGEs in glycated proteins with their role in diseases has been shown in Fig. 1.

Glycation and free radical theories of aging are used to explain the mechanism of aging [35]. According to glycation theory, cross linking and denaturation of proteins caused by glycation are the main factors for early aging induced alteration in tissues and blood vessels [44,45]. A recent study has put forward the alterations caused by biochemical side reactions as the essential mechanism of aging [83]. In this review, we shall discuss the evidences that support the role of glycated (AGE and Amadori products) lysine residues of proteins in aging and age-related diseases. Antigenicity of the glycated proteins and presence of autoantibodies against native and modified proteins, in age associated diseases, will be considered for the discussion.

2. Glycated lysine-rich proteins and their involvement in age-related diseases

There have been investigations on a number of proteins subjected to glycation [9]. Some of the important proteins were crystallin proteins [71], hemoglobin [13], proteins of erythrocyte membrane [42], insulin [19], human serum albumin (HSA) [67], high and low-density lipoproteins (HDL and LDL) [34], IgG [32, 39], IgM [41], collagen [23], histones [14,82]. Majority of these proteins were glycated through their ly-
sine residues. A recent investigation has found that in presence of cysteine residues, lysine residues react with RCS while arginine residues were protected from the reaction but in absence of cysteine the reaction mechanism was reversed [63]. Examples of glycated proteins (lysine rich) and products that are formed during the process of aging and age-related diseases have been summarized in Table 1.

Specific lysine residues in hemoglobin and human serum albumin have been identified as preferential sites of glycation in vivo [80]. In hemoglobin, the most reactive lysine residues appeared to be located to carboxylate groups in the primary or three-dimensional structure of the protein, while in albumin the reactive lysine is adjacent to another lysine residue in the primary sequence. In human body, histone proteins of the nucleus are richest in lysine and are modified to form CML [15]. Histones H3 and H2B were found to contain highest content of CML relative to lysine while histone H1 was the most highly carboxymethylated protein on a total protein basis. Glyceraldehyde induced Amadori modification of hemoglobin was reported by Acharya and Manning [1] and synthetic peptides were synthesized with lysine residues as the specific site for Amadori modification [22].

AGE modified proteins have been associated with aging and diabetes [53,78]. Apart from diabetes mellitus, AGEs are continuously linked to other age-related diseases like rheumatoid arthritis (RA) [16,40,43], Alzheimer [3,11]. Recent investigations have shown the role of AGEs, particularly CML, in aging and age associated risk of kidney damage and cardiovascular disease [26,65]. CML has been also detected in breast cancer [10,79] and plasma protein CML serves as a biomarker for age-related macular degeneration [52]. Glucosepane, a lysyl-arginine cross-link for collagen, is formed under non-oxidative conditions and is linked with aging and diabetes [46,66] while 2-Ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine), identified as AGE product of lysine in aged human lenses, is a potential risk factor for cataract [8].

Amadori- and AGE-modified histones were detected in liver cells of diabetic patients [29,30] whereas a number of Amadori-modified plasma proteins (viz. immunoglobulin heavy-chain constant regions) have been identified in type 2 diabetic patients [28]. Albumin
Table 1 Examples of proteins involving lysine residues for glycation and of glycated lysine product in age-related processes/diseases

| Proteins | Process/Disease | References |
|----------|----------------|------------|
| Collagen | Aging, Diabetes | Dunn et al. 1991, Sjoberg and Bulterij, 2009 |
| HDL & LDL CML | Diabetes mellitus, Aging, Atherosclerosis, Cancer | Kirstein et al. 1990, Schleicher et al. 1997, Bachmeier et al. 2008, Semba et al. 2009, Ni et al., 2009 |
| IgG | Diabetes mellitus and its complications, Rheumatoid arthritis | Kalia et al. 2004, Newkirk et al. 2003 |
| IgA | Diabetes mellitus and its complications | Kalia et al. 2004 |
| IgM | Diabetes mellitus and its complications | Kalia et al. 2004 |
| HSA | Diabetes mellitus and its complications, Alzheimer | Schalkwijk et al. 1999, 2002, Bouma et al. 2003, Cohen et al. 2010 |
| Histones | Diabetes mellitus | Jobst et al. 1991, Jobst and Lakattos 1996, Cervantes-Laurean et al., 2005, Kalia et al. 2004 |
| Hemoglobin | Diabetes mellitus and its complications | Akhtar et al. 1999, Ranjan et al. 2006 |
| Crystallins | Diabetes mellitus, Lens opacity | Akhtar et al. 1999, Ranjan et al. 2006 |

bearing Amadori compounds was associated with early nephropathy and with retinopathy in type 1 diabetic patients [59,60]. Glycated immunoglobulins (IgG, IgA, IgM) and glycated hemoglobin have been found in increased level in both type 1 and 2 diabetic patients with nephropathy as compared to the patients without any complications [31]. The investigations resulted in a detailed study for Amadori product in diabetes complications [47]. Previous reports on poly-L-lysine, a homopolymer of lysine residues with a large number of free ε-amino groups, were restricted to characterization of their browning products or AGEs [37,38]. In our investigation, the polypeptide was used to elaborate the role of Amadori lysine adducts in diabetes mellitus [5, 6].

3. Antigenicity of glycated lysine residues in proteins

Antihexitol lysine IgG, a polyclonal antibody raised in rabbits, was used to detect Amadori modified proteins in tissues of normal and diabetic rats [49] while Schalkwijk et al. [60] have used anti-Amadori albumin antibodies to recognize cyclic form (pyranose or furanose) of the Amadori product. Reactive carbonyl species, such as glyoxal, modify lysine and arginine residues of proteins but their reaction products (AGEs) were detected by antibodies specific for sugar-derived AGEs [58]. In our study, antigenicity of glycated lysine residues in proteins was probed by immunization of rabbits with glycated poly-L-lysine [5]. Glycated poly-L-lysine was found to be highly antigenic and the antigen exhibited a high degree of specificity for the induced antibodies. The study reflects that despite its homogeneous nature and lacking aromatic group, lysine polypeptide got the characteristic of an antigen when conjugated with glucose. The induced antibodies showed polyspecificity as they recognized similar modified lysine rich proteins (IgG, HSA and histones). This is the indication towards generation of common epitopes in the glycated lysine residues.

4. Serum autoantibodies against oxidized and glycated proteins in age-related diseases

A detailed study has been done for oxidatively modified autoantigens in autoimmune diseases [36]. Autoantibodies to native proteins such as GAD-65 and HSA are present in diabetes, but the antibodies to GAD-65 and other proteins have been reported both in patients with autoimmune diseases and in patients with cancer [12]. It has been shown that oxidized low-
Table 2
Activity of autoantibodies to glycated IgG in type 1 diabetes patients (Adapted from Rasheed et al. 2009)

| S. No. | Age (Year) | Sex (M/F) | Blood Glucose (mg/dl) | HbA1C | Smoking Duration | Detection of Anti-AGE-IgG Antibodies | Detection of Anti-IgG Antibodies | Cabonyl Contents (nmol/mg protein) |
|--------|------------|-----------|-----------------------|-------|-----------------|-------------------------------------|---------------------------------|----------------------------------|
|        |            |           |                       |       |                 | $A_{405}$ | MPI | $A_{405}$ | MPI | DM serum | DM-IgG |
|        |            |           |                       |       |                 | AGE-IgG | IgG | AGE-IgG | IgG |           |        |
| 1      | 60         | M         | 440                   | 8.9   | 32              | 0.92     | 65.1 | 0.49    | 25.5 | 3.5       | 1.3    |
| 2      | 61         | M         | 439                   | 8.8   | 34              | 0.93     | 64.3 | 0.48    | 27.2 | 3.6       | 1.1    |
| 3      | 61         | M         | 435                   | 9.2   | 36              | 0.94     | 64.4 | 0.53    | 27.2 | 3.5       | 1.2    |
| 4      | 60         | M         | 415                   | 8.2   | 34              | 0.93     | 62.2 | 0.54    | 21.1 | 3.3       | 1.1    |
| 5      | 57         | M         | 404                   | 8.8   | 33              | 0.89     | 57.4 | 0.46    | 32.3 | 3.1       | 1.0    |
| 6      | 59         | M         | 415                   | 8.5   | 30              | 0.81     | 54.2 | 0.43    | 29.4 | 3.5       | 1.2    |
| 7      | 59         | M         | 405                   | 7.8   | 29              | 0.90     | 53.3 | 0.37    | 30.2 | 3.0       | 1.1    |
| 8      | 41         | M         | 395                   | 7.4   | 16              | 0.89     | 51.5 | 0.36    | 29.0 | -         | -      |
| 9      | 40         | M         | 373                   | 8.2   | 13              | 0.89     | 52.6 | 0.37    | 35.8 | -         | -      |
| 10     | 40         | M         | 372                   | 7.8   | 15              | 0.85     | 49.0 | 0.35    | 38.4 | -         | -      |
| 11     | 39         | M         | 365                   | 8.1   | 9               | 0.84     | 43.8 | 0.31    | 33.0 | -         | -      |
| 12     | 34         | M         | 324                   | 7.1   | -               | 0.81     | 39.4 | 0.31    | 30.2 | -         | -      |
| 13     | 45         | F         | 329                   | 7.3   | -               | 0.71     | 41.4 | 0.37    | 31.3 | 2.6       | 0.9    |
| 14     | 25         | F         | 372                   | 8.5   | -               | 0.79     | 47.8 | 0.42    | 20.1 | -         | -      |
| 15     | 24         | F         | 364                   | 8.0   | -               | 0.77     | 37.1 | 0.41    | 31.8 | 2.9       | 1.0    |
| 16     | 58         | F         | 390                   | 8.6   | -               | 0.92     | 60.2 | 0.51    | 27.2 | 3.0       | 1.2    |
| 17     | 43         | F         | 352                   | 8.8   | -               | 0.90     | 43.1 | 0.37    | 20.1 | 2.7       | 0.8    |
| 18     | 35         | F         | 342                   | 7.2   | -               | 0.88     | 42.2 | 0.38    | 28.1 | -         | -      |
| 19     | 30         | F         | 340                   | 7.3   | -               | 0.86     | 37.1 | 0.39    | 19.4 | -         | -      |
|        | Mean±SD    |           | 45.8±13.0             | 12M/7F | 383±36.6 | 8.13±0.65 | 25.5±10.1 | 0.85±0.06 | 50.8±9.5 | 0.51±0.07 | 28.3±5.3 | 3.15±0.35 | 1.1±0.15 |
| Control| Mean±SD    |           | 42.0±15.0             | 14M/8F | 115±7.5   | 4.6±0.5   | 22.1±6.4  | 0.26±0.04 | 14.2±6.3 | 0.23±0.04 | 16.2±4.3 | 2.28±0.23 | 0.60±0.18 |

DM: type 1 diabetes mellitus; M: male; F: female; n: number of samples tested; DM-serum: serum from DM patients; DM-IgG: IgG from DM patients; NH-serum: serum from normal human; NH-IgG: IgG from normal human; $A_{405}$: absorbance at 410 nm calculated by direct binding ELISA; MPI: maximum percent inhibition at 20 mg/ml of inhibitor concentration calculated by competitive inhibition ELISA. *p<0.05 vs. $A_{405}$ (native IgG); **p<0.01 vs. MPI (IgG); ***p<0.05 vs. NH-serum; ****p<0.05 vs. NH-IgG.

density lipoproteins [20] and hydroxyl radical modified GAD proteins [33,75] were involved in production of autoantibodies in diabetic patients. Low levels of autoantibodies to amyloid beta peptide or oxidized LDL were associated with aging and risk factor for developing Alzheimer’s disease and atherosclerosis [70, 81].

Proteins modified by glycation and glycoxidation in vivo, serves as immunological epitopes leading to the production autoantibodies. Autoantibodies against AGE-HSA were detected in diabetic patients [76] whereas autoantibodies against glycated crystallins have been found in diabetic rats and cataract patients in the age group of 40–60 years [54,55]. Levels of these
autoantibodies were significantly higher at every stage when compared to their healthy controls. The concentration of antibodies was determined by antibody capture assay and that of antigen by non-competitive enzyme linked immunosorbent assay (ELISA). The studies of Shibayama et al. [68] and Araki et al. [7] have shown that the activity of autoantibodies to AGE especially CML increased with the duration of diabetic states. Patients with renal failure and those of diabetic nephropathy had a higher autoantibody activity than that in normal subjects or diabetic patients without any complication. They have put forward that immunocomplexes of autoantibody and AGE, accumulated in glomeruli, contribute to the pathogenesis of diabetes. Another study has shown that monoclonal antibodies against glycated albumin inhibit diabetic nephropathy [17]. Progression of diabetes to diabetic complications was found to be associated with the number of antibodies rather than a single antibody [72]. Although it remains unclear from the studies towards the role played by autoantibodies to glycated proteins in pathogenesis of the diseases, the autoantibodies can be used for prediction of autoimmune and age related diseases [64].

A previous investigation has found serum autoantibodies to AGE-IgG to the extent of 49% in rheumatoid factor positive RA patients [51]. Our investigation on role of glycated IgG in diabetes, revealed a binding of 61% of autoantibodies of type 1 diabetic patients to AGE-IgG as compared to the native form [57]. Reactivity of these serum autoantibodies to glycated IgG in the patients has been presented in Table 2. Sera of diabetic patients were probed for the presence of antibodies against glycated lysine residues with the use of glycated poly-L-lysine. Amadori-rich glycated poly-L-lysine was recognized by 64% of serum antibodies from diabetic patients (both type 1 & 2) [5]. The higher binding to glycated poly-L-lysine by the antibodies in diabetic patients suggests the involvement of glycated lysine residues in the production of autoantibodies in these patients.

### 5. Inhibition of glycated protein

The role of lysine residues in the glycation of proteins has been substantiated by the recent investigations showing inhibition of protein glycation by lysine [27,69]. Studies on several other glycation inhibitors have shown promising results in experimental animal models and some of them like GLY-230 (2-[3-chlorophenylamino][phenylacetic acid) decrease glycated albumin in diabetic patients while others like ALT-711 (3-phenacyl-4,5-dimethylthiazolium chloride), the AGE-cross-link breaker is in advanced clinical studies in humans [61]. The use of aminoguanidine is discontinued due to its side effect in humans but recently the use of pyridoxamine is also compromised [74]. Other studies have suggested that soluble receptor for AGE (sRAGE) or RAGE antagonist could be a future therapeutic target for inhibiting glycation [24]. The recent focus on bioactive substances of caffeic and chlorogenic acid [25] that are found in herbal mate tea and S-allyl cysteine in aged garlic extract [2] have shown positive effect towards inhibiting AGE formation.

The effect of glycation on lysine rich proteins and their involvement in aging and age-related diseases has been reviewed. The evidences support antigenicity of the glycated lysine residues in vivo with observation of autoantibodies against the glycated proteins in diabetes and RA patients. This could be due to protection of the modified proteins from proteolytic breakdown and its recognition as a foreign molecule by the immune system. A greater understanding of the regulation of glycated lysine products, especially Amadori products, in aging may play an important role in preventing the risk of age-related diseases. Thus, autoantibodies to glycated lysine rich proteins pose a marker for future age related diseases in presently healthy individuals.

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### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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