GLUCOSEPANE IS A MAJOR PROTEIN CROSS-LINK OF THE SENESCENT HUMAN EXTRACELLULAR MATRIX. RELATIONSHIP WITH DIABETES*

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Running Title: Major Cross-Link in Collagen

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The extracellular matrix (ECM) in most tissues is characterized by progressive age-related stiffening and loss of proteolytic digestibility that are accelerated in diabetes and can be duplicated by the nonenzymatic reaction of reducing sugars and ECM proteins. However, most cross-links of the Maillard reaction described so far are present in quantities too low to account for these changes. Here we have determined in human skin and glomerular basement membrane (GBM) collagen the levels of the recently discovered lysine-arginine cross-links derived from glucose, methylglyoxal, glyoxal, and 3-deoxyglucosone; i.e., glucosepane, MODIC, GODIC, and DOGDIC, respectively. Insoluble preparations of skin collagen (n=110) and glomerular basement membrane (GBM, n=28) were enzymatically digested and levels were measured by isotope dilution technique using liquid chromatography/mass spectrometry. In skin, all cross-links increased with age (p<0.0001) except DOGDIC (p=0.34). In nondiabetic controls levels at 90 yrs were 2000, 30, and 15 pmol/mg for glucosepane, MODIC and GODIC, respectively. Diabetes, but not renal failure, increased glucosepane to 5000 pmol/mg, (P<0.0001) and all others (< 60 pmol/mg, P<0.01). In GBMs, glucosepane reached up to 500 pmol/mg collagen and was increased in diabetes (P<0.0001) but not old age. In conclusion, glucosepane is the single major crosslink of the senescent extracellular matrix discovered so far, accounting for up to >120 mole % of triple helical collagen modification in diabetes. Its presence in high quantities may contribute to a number of structural and cell-matrix dysfunctions observed in aging and diabetes.

Reducing sugars react nonenzymatically with proteins to form adducts and cross-links referred as advanced glycation end products (AGEs)1(1). Their accumulation is particularly high in long-lived proteins, such as lens crystallins and collagen, and results in intra- and intermolecular cross-linking. The latter has been hypothesized to result in an age and diabetes-related stiffening of collagenous tissues and is believed to play an important role in the etiology of atherosclerosis and cardiovascular disease (3) as well as the loss of elasticity in lungs, joints, and skin (4-6). These age-related processes are markedly accelerated by diabetes and are associated with morbidity and mortality in diabetic individuals (7,8). In skin, protein cross-linking is associated with an age-related loss of elasticity, increased stiffening, and wrinkling (9). However, this process occurs ubiquitously with age suggesting a fundamental underlying mechanism.

While most of the age-related changes in the extracellular matrix (ECM) could be duplicated by incubating reducing sugars with proteins, Eble et al. (10) suggested that the major cross-links are not stable to conventional conditions of acid hydrolysis. Indeed, Biemel et al. (11) recently showed that glucose and sugar-derived dicarbonyl intermediates react with human serum albumin to form various acid-
labile lysine-arginine crosslinks, such as the 1,4-
dideoxy-5,6-glucosone-derived glucosepane, and the methylglyoxal-, glyoxal-, and 3-
deoxyglucosone-derived imidazolium crosslinks MODIC, GODIC, and DOGDIC, respectively (Fig. 1). An oxidized form of DOGDIC (DOGDIC-Ox) was also described (Fig. 1). Glucosepane was found in insoluble human lens protein reaching levels of 250 pmol/mg protein in some nondiabetic subjects (11). Levels of the others crosslinks were present in much lower quantities, i.e., < 75 pmol/mg for MODIC and < 5 pmol/mg for GODIC and DOGDIC.

In the present research, these cross-links were measured as a function of age in enzymatically digested human skin collagen and glomerular basement membrane (GBM) from both nondiabetic and diabetic subjects with and without renal failure or end-stage renal disease (ESRD).

Materials and Methods

Donor Tissue Information - Human skin and GBM were obtained as previously described (2) at autopsy from a total of n=110 individuals of which n=65 and n=45 were of the Caucasian and African-American race, respectively. There were n=56 males and n=54 females. A total of n=29 samples of GBM were used from the previous study (2). All samples were stored frozen in dry form at -80°C. Diagnoses of diabetes, renal disease, and other major pathologies and cause of death were obtained from each patient's medical and anatomical records at autopsy.

Preparation of Insoluble Collagen - Insoluble collagen was prepared as previously detailed (2,12). In brief, samples were delipidated, extracted with 1 M sodium chloride and 0.5 M acetic acid, digested with 0.1% pepsin in 0.5 M acetic acid at 4°C and freeze-dried (2).

Enzymatic Digestion of Insoluble Collagen - Approximately 5 mg of the freeze-dried pellet of insoluble skin or GBM was washed twice with 1 ml portions of buffer H (0.02 M HEPES and 0.1 M calcium chloride at pH 7.5; Sigma). The pellet was sequentially digested at 37°C for 24 hr consecutive intervals by the addition of the following enzymes in buffer H: 140 units (U) collagenase in 500 µl (Sigma); 118 mU peptidase in 25 µl (Sigma); 1 U pronase in 50 µl (Roche Applied Science); and 0.2 U aminopeptidase M in 10 µl (Roche Applied Science). Chloroform and toluene (1.5 µl) were added to every tube as antimicrobial agents. Digestion efficiency was determined by the ninhydrin reaction (13) and expressed as percentage of total leucine equivalents assayed in the hydrolysate. Digestibility varied from 100 to 41%, showing an inverse relationship as with age (r= -0.19, p=0.028).

Quantitative Analyses - The cross-links were determined in the digests as detailed by Biemel et al. (11). In short, samples (20 µl) were injected into a HP1100 (Agilent Technologies) HPLC system coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer (MS) equipped with an electrospray interface and a 150 X 4.6 mm YMC-Pack Pro C18 column at flow rates of 1 ml/min. Gradient separations consisted of water and methanol combinations using n-heptfluorobutyric acid as the ion-pairing agent (11). A postcolumn addition of propionic acid/2-propanol (3+1, v/v) in a 1: 3 ratio with the HPLC mobile phase was performed with a Knauer WellChrom Maxi-Star K-1000 HPLC pump. The MS parameters have previously been detailed by Biemel et al. (11). The quantification of the two isomers of DOGDIC-Ox (Fig. 1), was performed using 13C6-DOGDIC as the internal standard.

Statistical Analyses - Statistical methods including regression, correlation and ANOVA were performed as previously described by us (12,14,15) using SPSS software (SPSS Inc., Chicago, IL). Race, gender, presence of diabetes (type 1 and type 2), chronic renal disease (CRD) and end-stage renal disease (ESRD) were independent variables (16).

RESULTS

Effect of Age – Glucosepane significantly (p<0.0001) increased with age in nondiabetic subjects without renal failure reaching surprisingly high levels of nearly 2000 pmol/mg collagen at 90 yrs of age (Fig. 2A). Levels of MODIC, GODIC and DOGDIC-Ox also significantly (p<0.05) increased with age; however, reaching only about 30, 15, and 7 pmol/mg collagen at age 90 yrs, respectively.
Table I, Fig. 3A,B,D). DOGDIC reached ∼14 pmol/mg collagen, but did not increase with age (Table 1, Fig. 3C). Interestingly, the increased formation of DOGDIC-Ox correlated (r=0.56, p<0.0001) with an apparent decrease of DOGDIC, suggesting an enhanced oxidative medium in skin collagen (data not shown).

Effect of Diabetes – Diabetes significantly (p<0.006) increased levels of all cross-links (Table I). Glucosepane was elevated in many diabetic subjects far above the 95% confidence interval for controls reaching up to ∼4500 pmol/mg collagen (Fig. 2B). MODIC, GODIC, DOGDIC, and DOGDIC-Ox were also increased by diabetes reaching up to 55, 27, 32 and 9 pmol/mg collagen, respectively (Fig. 3A-D). Glucosepane (p=0.001), MODIC (p=0.041), GODIC (p=0.049) also increased with age in diabetics, but not DOGDIC (p=0.96) and DOGDIC-Ox (p=0.22).

Effect of Renal Failure – Multiple regression and ANOVA analyses showed that renal failure (p>0.20) had no effect on cross-link levels in both nondiabetic (p>0.20) and diabetic (p=0.13) subjects (Table I). However, since MODIC and GODIC tended to be higher in diabetic subjects with ESRD, the separate effects of ESRD were investigated. ANOVA analysis revealed that ESRD was significant for MODIC (p=0.018), GODIC (p=0.015) but only if untransformed data were used.

Effect of Race and Gender – Race had no effect (p>0.46) and therefore was deleted from subsequent analyses. Surprisingly, however, gender had a significant effect for both glucosepane (p=0.013) and GODIC (p=0.032) with diabetic females having higher levels than males by 62% for glucosepane (1933 vs. 1191 pmol/mg collagen) and 38% for GODIC (11.3 vs. 8.2 pmol/mg collagen). The Mann-Whitney test showed that the difference is statistically significant for both glucosepane (p=0.044) and GODIC (p=0.036).

Lysine-Arginine Crosslinks in Glomerular Basement Membranes – Glucosepane, MODIC, GODIC, and DOGDIC reached upward levels of approximately 500, 60, 80, and 20 pmol/mg collagen in GBM from nondiabetic donors, respectively (Fig. 4A-D). However, as previously observed for pentosidine (2), levels were in a steady state and did not increase with age (p>0.05). Glucosepane was significantly (p<0.0001) elevated by diabetes reaching levels over 900 pmol/mg collagen in one patient (Fig. 4A). Surprisingly, for the other crosslinks (Fig. 4B-D), diabetes did not elevate levels (p>0.05).

Estimated Contribution of the Amadori Product to Glucosepane Formation in Skin Collagen – Amadori products in skin collagen were estimated by furosine method assuming at 30% yield from acid hydrolysis (17). Assuming glucosepane forms according to the mechanism depicted in Fig. 1, the percent conversion of Amadori product into glucospane was determined as a function of age. As shown in Fig. 5A, this percentage increased with age (p<0.0001) reaching 43±16 % (SD) of the Amadori product at age 80 yrs. This percentage reached 53% in patients with type 2 diabetes and ESRD (p=0.024, Fig. 5B). Indeed, most data points for these patients lay outside the confidence intervals for controls (Fig. 5B). This suggests the existence of factors that either selectively catalyze glucosepane formation or impair the turnover rate of the collagen matrix.

DISCUSSION

In the present study, the non-fluorescent glucosepane levels increased up to approximately 2 nmol/mg collagen in old nondiabetic controls, and further increased up to 4.3 nmol/mg collagen in certain diabetic individuals (Fig. 2). This latter level represents about 1 to 1.2% (mol/mol) modifications in arginine and lysine residues, respectively. Assuming a molecular weight of 100,000 kDa for a single strand of triple helical collagen, this translates into approximate 1 cross-link for every 2 and 5 collagen molecules in diabetic and nondiabetic individuals, respectively. This result is much higher than that estimated for the number of pentosidine cross-links in cartilage by Verzijl et al.(18); i.e., 1 cross-link per 20 molecules of collagen. Surprisingly, this degree of cross-linking is within range of the lysyl oxidase-derived physiological cross-links where levels reach 1 to 5 mole/mole collagen in skin (19). The latter, however, do not increase with age (20,21). Furthermore, from the results of Vater et al.(22), it is estimated that this amount
of cross-linking induced by glucosepane should impose between a 30 to 45-fold decrease in collagen digestibility in diabetic skin.

In this study, substantial differences were noted in absolute levels of glucosepane between skin collagen versus GBM for both nondiabetic and diabetic individuals (Fig. 2 vs. Fig. 4). Specimens were not from the same individual, and are therefore not directly comparable. However, the most likely explanation for the differences in glucosepane levels is from differences in collagen turnover. First, although direct comparative measurements between skin and GBM are not available, work by Cohen and Surma (23) suggests that rat GBM turnover rate is as rapid as that of salt soluble Type I collagen which is notoriously poorly cross-linked. Another report suggests both GBM and tendon collagen turnover are at least greater than 100 days (24). Thus, while our data are compatible with higher turnover of GBM than skin, precise measurements are needed. Second, the extent of glycation of long-lived proteins like collagen is in equilibrium or steady-state relationship to ambient glucose concentration and further modulated by turnover (25-28). In that regard, the extent of glycation (Amadori product) is lower in GBM compared with tendon, aortic and skin collagen (29,30). Additionally, levels modestly but significantly increased with age in nondiabetic human skin collagen (26), but not GBM (31). Furthermore, GBM in the diabetic milieu undergoes increased collagen synthesis and thickening which in itself will affect turnover (30,32,33). Third, similar to the current study, previous work with the pentosidine cross-link showed that levels increased exponentially to over 90 pmol/mg collagen at age 90 yrs in nondiabetic skin collagen, while levels increased asymptotically to less than 50 pmol/mg, plateauing between the ages of 50 to 60 yrs in GBM from nondiabetics (2). Thus, these results further support the notion that GBM turns over at a faster rate compared with skin. Indeed, Verzijl et al. (28) determined the half-life of human skin collagen to be 15 yrs, although GBM was not studied.

The larger accumulation of glucosepane in skin collagen versus lens crystallins at late age; i.e., ~ 2000 (Fig. 2A) vs. 250 pmol/mg protein (11) is intriguing and may be due to the high concentration of methylglyoxal found in lens (34) which most likely competes with glucose for available modification sites. Indeed, the primary source of methylglyoxal is intracellular metabolism (35). In contrast, although not measured directly, methylglyoxal levels in ECM would be expected to be relatively lower as assessed by the low levels of methylglyoxal found in blood versus lens in normal healthy human subjects; i.e., ~ 80 vs. 2000 nM (34). In support, levels of the methylglyoxal-lysine dimer (MOLD) cross-link have also been found lower in nondiabetic human skin collagen versus lens; i.e., 0.38 vs. 8.0 mmol/mol of lysine at ages 85 and 70 yrs, respectively (36).

An additional observation in this study is that GBM levels of MODIC, GODIC and DOGDIC did not increase with age in diabetes (Fig. 4B-D), although such levels did increase in the skin with diabetes and aging (Fig. 3A-C). In a recent study measuring various antioxidant effects in diabetic and control rats (37), levels of these ODIC cross-links were found to be a mirror image of glucosepane formation in tendon collagen, thereby strongly suggesting that glucosepane and the ODIC crosslinks compete for the same modification sites on the collagen molecule, explaining why the much more predominant glucosepane masks the minor cross-links.

The above results also suggest that glucosepane is strongly correlated with the Amadori product in skin collagen and agree with the non-oxidative mechanism of glucosepane formation henceforth proposed by Biemel et al.(11) (Fig. 1). If furosine levels measured in these samples are considered (data not shown), and assuming a 30% yield of furosine from the Amadori product, it is estimated that as much as 50 to 60% of the steady state levels of Amadori product is converted into glucosepane at old age. The conversion is also significantly increased due to ESRD (p=0.024), but not diabetes alone, possibly because of decreased turnover rate of collagen in ESRD.

The largest level of any specific AGE measured in tissues so far has been the hydroimidazolones of methylglyoxal (34). Both glucosepane and the hydroimidazolones involve
arginine residues and are unstable to acid hydrolysis (11,38). In lens proteins, levels of the hydroimidazolone MG-H1 progressively increased with age reaching nearly 14 nmol/mg protein in old age (34) while glucosepane reached only 250 pmol/mg protein in old age (11). In contrast, the recently reported fluorescent lysine-lysine "K2P" cross-link reached 400 pmol/mg lens protein and up to 1400 pmol/mg protein in one brunescent lens sample (39). Neither the hydroimidazolones nor K2P have been determined in collagen. Thus, glucosepane is a relatively minor crosslink in lens but a major crosslink in collagen. It is intriguing to speculate that the high levels of the reversible methylglyoxal-derived hydroimidazolones may actually protect the lens crystallins from irreversible glucosepane-mediated damage.

An important question in diabetology is what role and extent does oxidative stress play in the diabetic milieu and its complications (40,41). Wells-Knecht et al. (42) previously reported that the age-dependent increase of two specific markers for oxidative stress in skin collagen; i.e., ortho-tyrosine and methionine sulfoxide, were surprisingly not accelerated by diabetes. However, in the present study, DOGDIC-Ox, also a marker for oxidative stress, was significantly elevated by diabetes (Table I, Fig. 3D). The apparent discrepancy may relate to differences in patient cohorts. Whereas the former study used a small sample size of diabetic individuals (n=12 to 17), a much larger number of subjects, some of which had severe complications, were included in this study (Table I).

Finally, levels of glucosepane and GODIC were found highly elevated in elderly postmenopausal women with diabetes (p<0.05, data not shown). The reason for the observed gender effect is not immediately apparent from this study. However, postmenopausal diabetic women are more at risk for cardiovascular disease, particularly coronary heart disease, an observation that has been well known for many years of uncertain etiology (43-48). Interestingly, it was reported that treatment of female rats with estradiol for one year attenuated the age-related increase in stiffening, glycoxidation, and permeability in carotid arteries (49). These findings suggests an inverse relationship between hormonal status and collagen crosslinking, a link suggested many years ago from the work of Everitt and associates using hypophysectomized rats (50,51).

In summary, this study reveals that glucosepane is the single major cross-link discovered to date in senescent human skin collagen and collagen from diabetic individuals, accounting for up to 120 mol% modification of triple-stranded helical collagen. It is also present in substantial quantities in GBM, although in a steady state. Its levels might be sufficient to contribute to the loss of digestibility of collagen in diabetes and aging. In fact, since at most 80% of old human collagen could be enzymatically digested, glucosepane levels could be even higher. The extent to which glucosepane is actually responsible for matrix stiffening and impairment of biologically critical arginine residues in the RGD sequences remains to be determined. From a pharmacological viewpoint, aminoguanidine was found to block the reaction sites on the 5,6-dioxo precursor compound (52), suggesting that the reported improvement in collagen solubility in diabetic animals receiving this drug (53-55) might in fact be due to covalent binding.

REFERENCES


**FOOTNOTES**

* This research was supported by Grants NIA AG18629 and NIDDK DK-57733 as well as a Mentorship Grant from the American Diabetes Association. We thank Ed C. Carlson (Dept. Anatomy and Cell Biology, University of North Dakota, Grand Forks, ND) for preparations of glomerular basement membrane from human kidneys.

The abbreviations used are: AGE, advanced glycation end-product; ECM, extracellular matrix; MODIC, methylglyoxal-derived imidazolium crosslink; GODIC, glyoxal-derived imidazolium crosslink; DOGDIC, 3-deoxyglucosone-derived imidazolium crosslink; DOGDIC-Ox, oxidized 3-deoxyglucosone-derived imidazolium crosslink; GBM, glomerular basement membrane; CRD, chronic renal disease; ESRD, end-stage renal disease

**FIGURE LEGENDS**

**Fig. 1.** Non-oxidative mechanism of glucosepane formation and structures of novel lysine-arginine crosslinks in skin collagen and GBM: glucose, methylglyoxal, glyoxal, and 3-deoxyglucosone-derived imidazolium cross-links, glucosepane, MODIC, GODIC, DOGDIC, and DOGDIC-Ox, respectively. Only selected isomers from each structure are displayed.

**Fig. 2.** Glucosepane levels in insoluble human skin collagen as a function of age, diabetes, and renal disease. (A) Nondiabetics with regression line and 95% confidence intervals: \(y=301e^{0.01x}\), \(r=0.78\), \(n=51\), \(p<0.0001\). (B) Diabetics with inserted regression line and confidence intervals computed for nondiabetic controls. Nondiabetic and diabetic individuals are represented by open and closed symbols, respectively:

- ○ nondiabetic
- □ nondiabetic with chronic renal failure (CRF)
- ▲ nondiabetic with end-stage renal disease (ESRD)
- ● diabetic (type 1 or type 2)
- ■ diabetic with CRF
- ▼ diabetic with ESRD

**Fig. 3.** Levels of the methylglyoxal, glyoxal, and 3-deoxyglucosone-derived imidazolium cross-links in insoluble human skin collagen as a function of age for diabetic individuals relative to the regression line and 95% confidence intervals of prediction determined for nondiabetics without renal failure. Data points for nondiabetics are not shown. See Fig. 2.

(A) MODIC, \(y= 6.13e^{0.01x}\), \(r=0.66\), \(n=51\), \(p<0.0001\);

(B) GODIC, \(y= 3.81e^{0.01x}\), \(r=0.66\), \(n=51\), \(p<0.0001\);
Fig. 4. Levels of lysine-arginine cross-links as a function of age in human glomerular basement membrane (GBM). Regression line and 95% confidence intervals were determined for nondiabetics. The effect of diabetes is significant for glucosepane (p<0.0001). See Fig. 2.
(A) Glucosepane, \( y=256 + 0.41x, r=0.08, n=21, p=0.72 \) (NS);
(B) MODIC, \( y=10 + 0.11x, r=0.28, n=19, p=0.24 \) (NS);
(C) GODIC, \( y=13 + 0.15x, r=0.23, n=19, p=0.36 \) (NS);
(D) DOGDIC, \( y=1.3 + 0.06x, r=0.34, n=19, p=0.16 \) (NS).

Fig. 5. Estimated percentage of glucosepane formation in skin collagen from the Amadori product calculated from furosine levels assuming a 30% yield (17). See Fig. 2. (A) Nondiabetics with regression line and 95% confidence intervals; \( y=11 + 0.36x, r=0.65, n=51, p<0.0001 \); (B) Diabetics relative to regression line and 95% confidence intervals for nondiabetics.
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<sup>a</sup> Superscript with asterisk indicates that the correlation is statistically significant (p<0.05).

<sup>b</sup> The number of individuals represented in this study was as follows: total, n=110; nondiabetics without renal failure, n=51; nondiabetics with renal failure, n=12; type 1 diabetics without renal failure, n=6; type 1 diabetics with renal failure, n=4; type 2 diabetics without renal failure, n=24; type 2 diabetics with renal failure, n=13.

<sup>c</sup> Does not include diabetes and renal failure.

<sup>d</sup> Renal failure is individuals with either chronic renal failure (CRF) or end stage renal disease (ESRD) as previously defined (2).
Figure 1

Glucose → Amadori Product → 1,4-Dideoxy 5,6-glucosone → Glucosepane

GODIC MODIC DOGDIC DOGDIC-Ox
Figure 3

(A) MODIC

(B) GODIC

(C) DOGDIC

(D) DOGDIC-OX

Collagen Cross-link (pmol/mg collagen) vs. Age (years)
Figure 4

(A) Collagen Cross-link (pmol/mg collagen)

(B) Glucosepane

(C) GODIC

(D) DOGIC

Age (years)
Figure 5

(A) % Glucosepane Formation from the Amadori Product (nmol/nmol) *100

(B) % Glucosepane Formation from the Amadori Product (nmol/nmol) *100

Age (years)
Glucosepane is a major protein cross-link of the senescent human extracellular matrix. 

Relationship with diabetes

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*J. Biol. Chem.* published online January 26, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500733200

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