

# Effects of Age and Diabetes Mellitus on the Solubility and Nonenzymatic Glucosylation of Human Skin Collagen

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**ABSTRACT** Collagen from human skin was fractionated into neutral salt-soluble, acid-soluble, pepsin-released, and insoluble fractions. No age-related changes were observed in the proportion of collagen extracted by neutral salt. A significant age-related decrease in the proportion of acid-soluble collagen was found. A highly significant ( $P < 0.001$ ) age-related decrease in the amount of collagen released by pepsin digestion was observed, with a concomitant age-related increase in the fraction of insoluble collagen. The amount of ketoamine-linked glucose bound to this insoluble collagen also increased significantly with age.

Skin collagen from three juvenile onset diabetics (JOD) and one young maturity onset diabetic (MOD) appeared to have undergone accelerated aging. JOD and the young MOD had significantly less collagen released by pepsin digestion and significantly more insoluble collagen than would be predicted by their ages. The collagen released by pepsin digestion of the diabetic samples had more high molecular weight components than similar fractions obtained from age-matched nondiabetic controls. There was also more ketoamine-linked glucose bound to the insoluble collagen of JOD than to that fraction from comparably aged control subjects. The apparent acceleration of collagen aging in diabetes mellitus may play a role in complications of diabetes that occur in collagen-rich tissues.

## INTRODUCTION

Collagen normally undergoes a progression of age-related changes characterized by decreasing solubility, increasing stiffness, and resistance to enzymatic digestion (1, 2). Evidence has been obtained from collagenase digestion studies suggesting that collagen from diabetics is prematurely aged (3-5).

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Since many of the complications affecting diabetics resemble debilities of age and occur in collagen-rich tissues, it has been suggested that accelerated aging of collagen in the diabetic may play an important role in their pathogenesis (3). Among these complications are an earlier onset and greater severity of atherosclerosis (6), premature stiffening of large blood vessels (7), diffuse thickening of capillary basement membranes (8), and diminished elastic recoil of lungs (9).

The present study was undertaken to extend previous observations of aging phenomena occurring in collagen of human tendon (10-13), dura mater (11, 12), and myocardium (14), and to investigate further the apparent accelerated aging of collagen in human diabetes. Skin was chosen for this study because it is a tissue rich in collagen (2, 15) as well as the site of a number of age-related changes (16), and particularly because it can be a useful source of biopsy material for future studies in living subjects.

A systematic fractionation and characterization of human skin collagen, as a function of age and in the presence or absence of diabetes mellitus, was carried out. The amount of recently synthesized collagen was determined by measuring the amount of collagen extracted by neutral salt solutions (17, 18). Collagen soluble in dilute acetic acid was also determined. This fraction is somewhat more mature and contains more covalent crosslinks than neutral salt-soluble collagen (18). Pepsin digestion was used to obtain a third fraction. This treatment causes solubilization of collagen, presumably by degrading the nonhelical telopeptide regions of the collagen molecule (19), where crosslinking is known to occur (20), while leaving the helical portion intact (19). Collagenous components in the pepsin-released fraction were analyzed by polyacrylamide gel electrophoresis. The insoluble collagen remaining represents the most densely cross-linked fraction (18).

The amount of ketoamine-linked glucose bound to the insoluble collagen fraction was measured to determine if an age- and diabetes-related increase in this

glucosylation occurs in skin collagen as it does in insoluble human tendon collagen (13).

## METHODS

Samples of abdominal or thoracic skin were obtained at autopsy from 21 subjects without clinical or pathological evidence of connective tissue disease or diabetes mellitus, from 3 subjects with juvenile onset diabetes (JOD),<sup>1</sup> and from 3 subjects with maturity onset diabetes (MOD). The diagnosis of diabetes was based on criteria established by the National Diabetes Data Group (21).

**Extraction procedure.** The skin samples were immediately frozen at  $-70^{\circ}\text{C}$  until used. Frozen samples were thawed and rinsed with tap water. Epidermis and subcutaneous fat were scraped off. The remaining tissue was blotted dry, and  $\sim 0.75$  g were used for further processing. Samples were homogenized in 1.0 M NaCl, 0.05 M Tris-HCl buffer (pH 7.4) with a polytron, until all of the tissue was in a thick suspension. Suspensions were stirred for 18 h at  $4^{\circ}\text{C}$  and centrifuged at 11,000 g for 20 min at  $4^{\circ}\text{C}$ . The precipitates were suspended twice in the neutral salt solution and recentrifuged. Supernatant solutions were combined. Collagen in supernatant solution is referred to as the neutral salt-soluble fraction.

The precipitate was extracted in an 0.5 M acetic acid solution with stirring for 18 h at  $4^{\circ}\text{C}$ . The suspension was centrifuged at 11,000 g for 20 min at  $4^{\circ}\text{C}$ . The precipitate was washed with 0.5 M acetic acid and recentrifuged. Collagen in the combined supernatant solution is referred to as acid-soluble collagen.

The precipitate from 0.75 g of skin remaining after the acid extraction was suspended in 20 ml of 0.5 M acetic acid and digested with pepsin (Worthington Biochemicals, Freehold, N. J.) at a concentration of 1.0 mg/ml for 18 h with stirring at  $4^{\circ}\text{C}$ . NaCl in Tris-HCl buffer (pH 7.4) was added to the viscous digestion mixture after the incubation was completed, to a final salt concentration of 0.17 M. Addition of salt was found to aid in precipitation of collagen from the viscous digestion mixture. Only one digestion was carried out, because the purpose was to obtain a measure of collagen stabilization rather than to obtain a maximal yield of solubilized collagen. Collagen solubilized after pepsin digestion was collected by centrifuging the mixture at 50,000 g for 1 h at a rotor temperature of  $4^{\circ}\text{C}$ . The precipitate was suspended twice in the neutral salt solution and centrifuged. Supernatant solutions were combined. Collagen solubilized in this way is referred to as the pepsin-released collagen fraction.

The collagen remaining after the two extraction procedures and the pepsin digestion is considered insoluble. The insoluble collagen was washed twice with distilled water and centrifuged at 75,000 g for 20 min at a rotor temperature of  $4^{\circ}\text{C}$ . The resulting precipitate was suspended in distilled water by homogenizing in a glass homogenizer.

**Measurement of collagen.** Collagen present in these fractions was estimated by measuring the amount of hydroxyproline. Hydroxyproline was assumed to comprise 14% of the collagen by weight (10). Samples were hydrolyzed in 6.0 N HCl for 16 h in an autoclave. The HCl was removed *in vacuo* and the amount of hydroxyproline was determined by a modification of the method of Stegeman and Stalder (22).

<sup>1</sup>Abbreviations used in this paper: JOD, juvenile onset diabetic; MOD, maturity onset diabetic; PAGE, polyacrylamide gel electrophoresis.

**Quantification of ketoamine-linked glucose.** Glucosylation of insoluble collagen was measured by the thiobarbituric acid method as modified by Pecoraro et al. (23). The amount of skin collagen that was glucosylated was found to be low in preliminary determinations. We further modified the technique to enable us to use larger amounts of collagen for the measurement of ketoamine-linked glucose: an equal volume of 1.0 M oxalic acid was added to aliquots of homogenized insoluble skin collagen. The mixture was incubated for 4.5 h at  $100^{\circ}\text{C}$ , cooled to room temperature, and centrifuged at 300 g for 20 min at  $4^{\circ}\text{C}$ . The supernate was lyophilized, suspended in 2 ml of distilled water, and filtered through Whatman 42 filter paper (Whatman Inc., Clifton, N. J.). The amount of ketoamine-linked glucose in the filtrate was measured as previously described (23). Although glycosidically-bound glucose is present in the insoluble collagen samples, it is unlikely that this carbohydrate will be converted to a furfural under the conditions of this assay (24). Fructose was used as a standard. The amount of 5-hydroxymethylfurfural was determined by a conversion factor determined with pure 5-hydroxymethylfurfural (Sigma Chemical Co., St. Louis, Mo.).

**Analysis of collagen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** Collagen type and an estimate of its crosslinking were determined by SDS-PAGE. The procedure used to distinguish between type I and III collagen was a modification of the method of Sykes et al. (25). Samples of the pepsin-released fraction were dialyzed in a 3,200-dalton cut-off Spectropor membrane (Spectrum Medical Industries, Inc., Los Angeles, Calif.) against an 0.0625 M Tris-HCl (pH 6.8), 0.002 M EDTA buffer. The retentate was lyophilized. The lyophilized material was then suspended in a sample buffer composed of 0.0625 M Tris-HCl (pH 6.8), 0.002 M EDTA, 2.3% (wt/vol) SDS, and 10% (vol/vol) glycerol, and heated at  $60^{\circ}\text{C}$  for 30 min.

The SDS-PAGE slab gels were composed of a 1-cm high 10% (wt/vol) acrylamide gel base, an 8-cm high main gel of 6% acrylamide, and a 2.5-cm high stacking gel of 4.5% acrylamide. The 10% gel base and 6% main gels contain 1.08% (wt/vol) *N,N'*-methylene-bis-acrylamide (BIS), 0.125 M Tris-HCl (pH 8.8), 0.1% (wt/vol) SDS, 0.1% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 0.1% (wt/vol) ammonium persulfate. The 4.5% stacking gel contains 1.08% (wt/vol) BIS, 0.125 M Tris-HCl (pH 6.8), 0.1% (wt/vol) SDS, 0.1% (wt/vol) TEMED, and 0.1% (wt/vol) ammonium persulfate.

Between 25 and 50  $\mu\text{g}$  of collagen was applied to each well, and electrophoresis was carried out for 1 h with a constant current of 40 mA/gel at room temperature. The current was turned off and each well was filled (25–50  $\mu\text{l}$ ) with 10% (vol/vol) 2-mercaptoethanol in sample buffer. After letting the mercaptoethanol diffuse into the gel for 1 h the current was turned on and electrophoresis was continued until the tracking dye reached the bottom of the 10% gel base. Gels were stained with Coomassie Brilliant Blue (R-250) for 10 min and destained overnight in 30% (vol/vol) methanol and 7% (vol/vol) acetic acid. Specific localization of collagen bands was accomplished by the collagen-specific staining technique of McCormick et al. (26).

Gels were photographed with a Polaroid MP4 Land camera with Kodak Contrast Process Pan film, through an orange filter (Wratten 21) to optimize the film's blue sensitivity. Photographic negatives of the gels were scanned in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a linear transport device.

**Statistical methods.** Skin samples were processed and all assays were performed without knowledge of the age or clinical status of individual subjects. Linear equations, regression coefficients, and confidence bands were generated by a linear regression program with a calculator. Formulae for

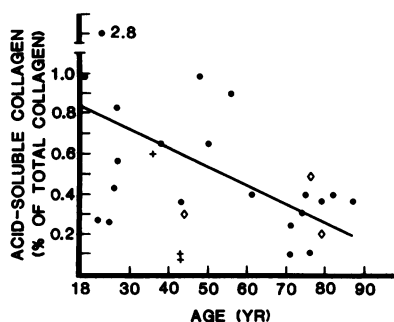


FIGURE 1 The proportion of collagen extracted by 0.5 M acetic acid is plotted as a function of subject's age. The solid line represents the regression equation  $y = -0.0107x + 1.14$ ,  $r = -0.44$ ,  $P < 0.05$ , and is derived from the data of nondiabetics. No confidence bands are plotted because of the large variability among young mature and middle aged subjects. Nondiabetic subjects ( $\bullet$ ,  $n = 21$ ); JOD (+,  $n = 3$ ); MOD ( $\diamond$ ,  $n = 3$ ).

determining confidence bands were obtained from Armitage (27). Probability values were determined from tables in Documenta Geigy (28).

## RESULTS

Relatively small amounts of collagen were recovered from the neutral salt- and acid-soluble fractions. There was no apparent relationship between the proportion of recently synthesized, neutral salt-soluble collagen, and age or the presence or absence of diabetes.

There was a statistically significant decrease with age ( $r = -0.44$ ,  $P < 0.05$ ) in the proportion of collagen extracted by 0.5 M acetic acid among the nondiabetic subjects (Fig. 1). Although the slope of this line is negative and the probability of the correlation coefficient is significantly different from zero, there is considerable variability of values, especially in the younger age groups. Two of the JOD had significantly ( $P < 0.05$ ) less acid-soluble collagen than nondiabetics of comparable ages.

The proportion of collagen solubilized by pepsin digestion of the salt and acid-insoluble collagen was significantly decreased as a function of age (Fig. 2,  $r = -0.809$ ,  $P < 0.001$ ).

All JOD and one young MOD had significantly ( $P < 0.001$ ) less collagen solubilized by this digestion procedure than the age-matched nondiabetics. Two older MOD, however, did not have less collagen released by this treatment than comparably aged nondiabetics.

Age-related differences in the electrophoretic patterns of the collagen solubilized after pepsin digestion were not consistent, although there was a trend for increased amounts of higher molecular weight collagen components with increasing age. More high molecular weight collagen components were consistently observed

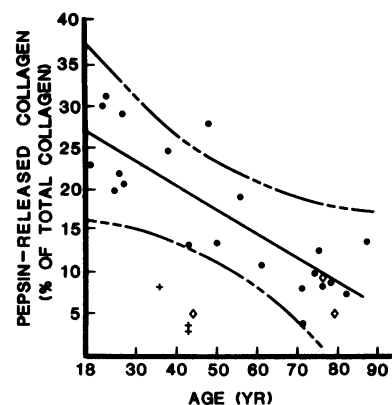


FIGURE 2 The proportion of collagen released after pepsin digestion is plotted as a function of subject's age. The solid line represents the regression equation  $y = -0.295x + 32.04$ ,  $r = 0.809$ ,  $P < 0.001$ . The 99.5% confidence bands are represented by the dashed lines. The regression line and confidence bands are derived from the data on nondiabetics. The symbols are the same as in Fig. 1.

in gel scans of young diabetics than in those of age-matched nondiabetics (Fig. 3).

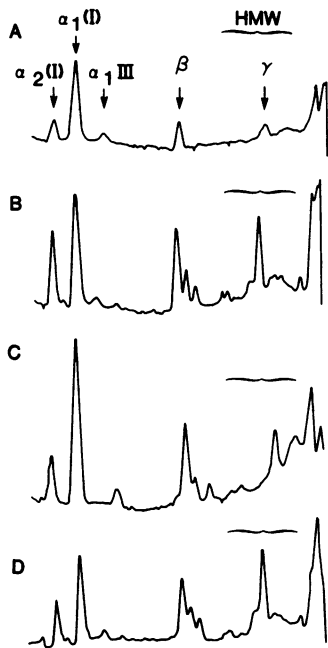
The proportion of collagen in the insoluble fraction was positively and significantly associated with subject age ( $y = 0.412x + 57.94$ ;  $r = 0.804$ ;  $P < 0.001$ ). The proportion of insoluble collagen from JOD and a younger MOD was also significantly ( $P < 0.001$ ) greater than would have been predicted by the subjects' ages. The two older MOD did not have values exceeding nondiabetics of comparable ages.

The amount of ketoamine-linked glucose in the insoluble collagen fraction was significantly correlated with subject age (Fig. 4,  $r = 0.662$ ,  $P < 0.01$ ). The insoluble collagen of JOD contained from 2-3 times more ketoamine-linked glucose than corresponding age-matched controls ( $P < 0.001$ ). MOD, however, did not appear to have increased amounts of ketoamine-linked glucose bound to their insoluble collagen.

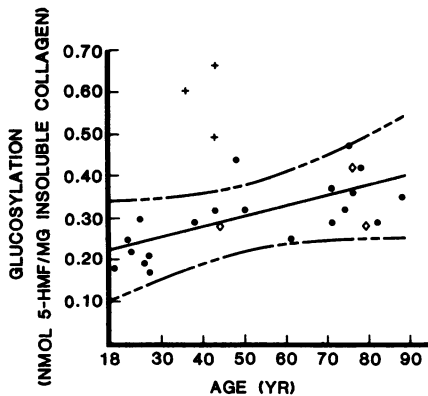
## DISCUSSION

One 48-yr-old nondiabetic subject had substantially more pepsin-released collagen and less insoluble collagen than other similarly aged nondiabetics (Fig. 2). This subject's clinical history was significant for multiple organ system disease including cerebral palsy, schizophrenia, hypertension, and undifferentiated sarcoma. One possible explanation for his outlying values may be that the skin used in this study was irradiated during treatment for the sarcoma. Collagen from irradiated areas is actinically damaged and known to be more soluble (15).

The proportion of collagen extracted by neutral salt and dilute acid solutions comprised a small fraction of the total collagen from all subjects. This reflects



**FIGURE 3** SDS-PAGE scans of pepsin-released collagen reduced *in situ*. The tracings are from photographic negatives of gels. (A) 48-yr-old nondiabetic subject; (B) 43-yr-old JOD; (C) 36-yr-old JOD; (D) 44-yr-old MOD. The material remaining at the top of the gel is composed of high molecular weight collagen. The next highest molecular weight material is from trimers of collagen polypeptide chains, which are labeled as gamma chains ( $\gamma$ ); dimers of collagen polypeptide chains are labeled as beta chains ( $\beta$ ). The fastest migrating collagen components are polypeptide monomers labeled as alpha chains ( $\alpha$ ). High molecular weight (HMW) collagenous material that is increased in diabetics is indicated by the brackets.



**FIGURE 4** The amount of ketoamine-linked glucosylation of insoluble collagen is plotted as a function of subject's age. The solid line represents the regression equation  $y = 0.0025x + 0.179$ ,  $r = 0.662$ ,  $P < 0.01$ . The 99.9% confidence bands are represented by the dashed lines. The regression line and confidence bands are derived from the data on nondiabetics. The symbols are the same as in Fig. 1. 5-HMF, 5-hydroxymethylfurfural.

the slow rate of collagen synthesis following cessation of growth (1, 17, 18). We observed an age-related decrease in the proportion of acid-soluble collagen, but no age-related change in the proportion of neutral salt-soluble collagen. Similar age-related decreases in acid soluble collagen have been reported in human tissues (1, 2, 16, 17, 29) and in mouse skin (22). Likewise, the absence of an age change in the amount of neutral salt-soluble collagen extracted from human skin has been previously reported (30). Two of the three JOD had less acid-soluble collagen compared with comparably aged nondiabetics. A similar decrease in the proportion of acid-soluble collagen extracted from dermal granulation tissue of streptozotocin-induced diabetic rats has been recently reported (31). The effect of diabetes on the reduced solubility of diabetic collagen was reversed by treatment of the diabetic rats with inhibitors of lysyl oxidase-dependent covalent cross-linking of collagen (31).

Collagen from human skin became increasingly resistant to the solubilizing effects of pepsin as a function of age. Collagen from human tendon, dura, and myocardium has been shown to become progressively resistant to digestion by collagenase with increasing age (11, 12, 14).

Increased amounts of high molecular weight collagen components from pepsin-released samples of older nondiabetics were not consistently observed in SDS gels. There was, however, a general trend for their appearance with increasing age. Young diabetics had consistently more high molecular weight collagen components released by pepsin digestion. These components were not labile to extensive SDS and heat denaturation, or to mercaptoethanol reduction. This suggests the presence of covalent interaction between collagen  $\alpha$ -chains. Collagen from young diabetics may be more resistant to these denaturing and reducing conditions because of an abnormal covalent interaction or a higher concentration of normal covalent interactions. A similar increase in high molecular weight collagen components has been reported in extracts of rat tail tendon (32) and dermal granulation tissue (31) from streptozotocin- or alloxan-induced diabetic rats, as compared with nondiabetic controls. A greater covalent interaction of collagen molecules in diabetes is supported by the observation that collagen from JOD is much more resistant to collagenase digestion than collagen from even the oldest nondiabetic subjects (4).

The amount of ketoamine-linked glucose bound to insoluble collagen also increased as a function of age. This increase was not as dramatic as previously reported for insoluble human tendon collagen (13). The difference may be due to the higher turnover rate of skin collagen compared with tendon collagen (17). The nonenzymatic glucosylation of other proteins has

been shown to be a function of the duration of exposure and concentration of glucose in the environment (33, 34). Thus, if skin collagen is turned over more rapidly than tendon collagen, the opportunity for the former to become glycosylated will be diminished. The amount of ketoamine-linked glucose bound to insoluble collagen was markedly increased in JOD. A similar increase was previously reported for tendon collagen from JOD (13).

The collagen from a young MOD was similar to the collagen of JOD. Collagen from older MOD, however, did not differ significantly from older nondiabetics. Such differences between diabetics may be manifestations of diverse etiologies and pathologies of maturity onset diabetes (21, 35). It is also possible that collagen in the older MOD's may have become maximally glycosylated, but at an earlier age. This could also be the case for nonenzymatic glycosylation (13) and collagenase digestion (3) of human tendon collagen from old diabetics.

Data in the present study are therefore consistent with the notion that individuals with diabetes mellitus, particularly JOD have accelerated aging of their collagen (3, 4). The cause of this apparent accelerated aging, however, is not known. Three hypotheses merit consideration. The first is that an altered metabolic environment causes secondary changes in collagen leading to the observed accelerated aging phenomena. The hyperglycemic environment of the diabetic probably leads to increased nonenzymatic glycosylation of proteins (33, 34). There is an increased glycosylation of insoluble collagen from tendon (13) and skin of human diabetics. There are also reports of increased glycosylation of a collagenous fraction isolated from glomerular basement membrane of human diabetics (36) and from rats with streptozotocin-induced hyperglycemia (37). It has also been reported that nonenzymatic glycosylation of rat aortic collagenous material is increased in streptozotocin-induced hyperglycemia (38). It has not been demonstrated, however, that glycosylation of collagen results in the observed altered properties of collagen occurring with age and accelerated in diabetes (i.e., solubility and collagenase digestibility).

A second hypothesis is that abnormal collagen is synthesized by diabetics and that this abnormality leads to changes that appear to represent accelerated aging. It has been reported that fibroblasts from JOD in vitro secrete an abnormal procollagen molecule that lacks normal nonreducible crosslinks and contains abnormal reducible crosslinks (39). The collagen secreted by cells from diabetics is also excessively soluble (40). This excessive solubility may be due to abnormal crosslinks that inhibit normal proteolysis of procollagen. Alternatively, it is possible that the altered metabolic environment of diabetics selects for certain fibroblast cell lines that are normally present in only small num-

bers. These fibroblasts may synthesize an abnormal collagen molecule. Changes that have been observed in the collagen of diabetics might cause the characteristic complications of aging that occur in collagen-rich tissues to develop at an earlier age.

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