# Rapid Publications

# Glucosylated Collagen Is Antigenic

A. R. BASSIOUNY, H. ROSENBERG, AND T. L. McDONALD

**SUMMARY**

**Sera from rats inoculated with in vitro glucosylated rat skin acid-soluble collagen were evaluated for specific antibody response using the enzyme-linked immunosorbent assay. The data indicate that the altered protein is capable of eliciting the production of antibodies, but unmodified collagen is not. The specificity of the generated antibodies appears to be directed toward the glucitollysine residues of the modified collagen. Sera from streptozotocin-induced diabetic rats contain antibodies that clearly bind glucosylated collagen.**

**These results provide support for the hypothesis that in diabetes mellitus increased collagen glucosylation during chronic hyperglycemia may be the initiating event of an autoimmune response leading to the long-term complications. DIABETES 32:1182–1184, December 1983.**

Fresh insights into the development of diabetic macroangiopathy and microangiopathy have emerged due to the demonstration of excessive nonenzymatic glucosylation of proteins in the diabetic state.[1] Available evidence suggests that collagen, the predominant protein of the body, is a major target for attachment of glucose by means of a stable ketoamine linkage involving the ε-amino groups of lysine and hydroxylysine residues.[2] Indeed, an increase in nonenzymatic glucosylation of skin collagen,[3,4] aortic collagen,[5,6] and glomerular basement membrane collagen[7,8] has been demonstrated in diabetic humans and diabetic laboratory animals. Since the formation of collagen cross-links is attributable primarily to the presence of lysine and hydroxylysine residues, it has been speculated that the increased substitution of glucose on these residues may result in abnormal cross-linking and hence structural and functional changes in the protein leading to the vascular complications of diabetes.[8] In addition to the possible alteration of the functional properties of collagen, excessive nonenzymatic glucosylation may render the protein antigenic and thereby initiate an autoimmune response contributing to the development of vascular complications.

In the present article, we extend our studies on the nonenzymatic glucosylation of collagen by examining the antigenicity of in vitro glucosylated rat skin acid-soluble collagen in allogenic animals.

From the University of Nebraska Medical Center, Omaha, Nebraska.
Address reprint requests to Dr. Harry Rosenberg, The University of Nebraska Medical Center, College of Pharmacy, 42nd and Dewey, Omaha, Nebraska 68105.
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## METHODS AND MATERIALS

**Extraction of acid-soluble collagen from rat skins.** Acid-soluble collagen was extracted from skins obtained from normal 6-mo-old rats (Sprague-Dawley-derived strain) according to the procedure described by Rauterberg and Kuhn.[9] Fresh minced rat skins were extracted three times with several volumes of 0.5 M sodium acetate solution containing 0.1% EDTA as the disodium salt. These extracts were discarded while the residue was further washed with 0.01% EDTA followed by extraction with several volumes of 0.05 M citrate buffer, pH 3.7, containing 0.05 M NaCl. The insoluble residue was collected by centrifugation and discarded. The soluble collagen was precipitated by the addition of concentrated NaCl solution to the supernatant and then collected by centrifugation, redissolved in 0.1% acetic acid, and again precipitated with NaCl. This precipitate was again dissolved in 0.1% acetic acid, exhaustively dialyzed against 0.1% acetic acid, and subsequently centrifuged for 4 h at 32,000 × *g* in order to remove all particulate matter. For storage the collagen solution was lyophilized.

**In vitro glucosylation of rat skin collagen.** Acid-soluble rat skin collagen was dissolved in 0.2 M phosphate buffer (4 mg/ml), pH 8.0, and incubated with 40 mM glucose in the presence of 40 mM $NaCNBH_3$. The preparation was kept at 37°C with occasional shaking. After 6 days, the incubation was dialyzed in the cold against six changes of 1000-fold volumes of distilled water and finally lyophilized.

TABLE 1
Detection* of antibodies reactive with glucosylated collagen in the sera of immunized rats

| ELISA coating antigen | Antigen administered† | | | | | | |
|---|---|---|---|---|---|---|---|
| | Glucosylated collagen | | | | | | Unmodified collagen (all six rats) |
| | 1‡ | 2‡ | 3‡ | 4‡ | 5‡ | 6‡ | |
| Glucosylated collagen | 6,400 | 12,800 | 1,600 | 1,600 | 3,200 | 800 | 0 |
| Unmodified collagen | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Quantitation of antibody was performed by ELISA titration of sera against either glucosylated or unmodified collagen coated onto plates at 1 μg/well.
†Six rats were injected with either glucosylated or nonglucosylated rat skin collagen as described in METHODS AND MATERIALS.
‡Animal number.

The presence of glucitollysine (the amino acid derivative that resulted from the binding of glucose to the ε-amino group of lysine) in the glucosylated collagen was determined on a Beckman 120 C Amino Acid Analyzer (Beckman Instruments, Fullerton, California) after hydrolysis of collagen samples in 3 N HCl at 110°C for 24 h. Synthetic glucitollysine (prepared by the method of Trueb et al.[10]) served as a standard in the identification of the amino acid derivative in glucosylated collagen.

**Antigenicity testing.** Young adult rats (Sprague-Dawley-derived strain), weighing 100–120 g, were each pretreated with an intravenous injection of levamisole HCl (Sigma Chemical Company, St. Louis, Missouri) at a dose of 12 mg/kg in order to enhance the immune response. Solutions containing in vitro glucosylated and nonglucosylated rat skin collagen (4 mg protein/ml in 0.2 M phosphate buffer, pH 7.4) were mixed with one volume of Freund's complete adjuvant before intraperitoneal inoculation of animals. One group of six animals each received 1 mg of glucosylated rat skin collagen, while another like group received an identical dose of unmodified collagen. Three and five weeks later, the animals were boosted with 1-mg quantities of the appropriate collagen solutions mixed with 1 vol of Freund's incomplete adjuvant.

Sera were collected from all experimental animals 2 wk after the last booster injection and were subsequently analyzed for specific antibody by the indirect enzyme-linked immunosorbent assay (ELISA). In vitro glucosylated and nonglucosylated rat skin collagen were used as plate-coating antigens (1 μg/well). One hundred microliters of twofold dilutions of test sera were then added to triplicate wells of microtitration plates (Dynatech Laboratories, Alexandria, Virginia). After suitable washing, an alkaline phosphatase conjugate of anti-rat IgG (Miles Laboratories, Elkhart, Indiana) was incubated in the wells followed by the addition of enzyme substrate (p-nitrophenyl-phosphate). The endpoint titers were determined by the reciprocal of the highest dilution of sera that had a color absorbance greater than 0.200 at 405 nm.

In order to determine the epitope specificity of the antibodies generated against glucosylated collagen, test sera were incubated with glucitollysine (1 mg/ml) for 1 h at 37°C before ELISA testing.

**Detection of antibody to in vitro glucosylated rat skin collagen in sera of streptozotocin-diabetic rats.** Young rats, weighing 120–130 g, were rendered diabetic by a single tail vein injection of streptozotocin (60 mg/kg). Only rats that developed persistent polydypsia, polyuria, and glycosuria and had blood glucose levels in excess of 350 mg/dl were used. The diabetic state was monitored by measuring urine glucose with Tes-Tape (Eli Lilly Co., Indianapolis, Indiana) and blood glucose with the glucose-oxidase method (Sigma Glucose Oxidase Kit, Sigma Chemical Co.).

The presence of antibody capable of binding to in vitro glucosylated rat skin collagen in the sera of rats with acute diabetes of 5 mo duration was determined by the ELISA procedure.

## RESULTS

The data in Table 1 show the antibody titers that were detected in the sera of rats inoculated with glucosylated rat skin collagen. Although the concentration of antibody with specificity for glucosylated collagen was different for individual rat sera, none of the sera were reactive with nonglucosylated rat skin collagen. These data imply that glucosylation was the initiator for antigenicity and not merely a possible alteration of collagen during the extraction procedure. This contention is supported by the antibody unresponsiveness of six rats inoculated with unmodified rat skin collagen. Antibody activity to either glucosylated or nonglucosylated collagen was not detected in these rats by ELISA (Table 1).

In order to determine the epitope specificity of the rat antibody produced in response to glucosylated rat skin collagen, positive sera from one animal were diluted 1:400, preincubated with 1 mg/ml glucitollysine, and reacted in ELISA plates coated with glucosylated collagen. The results show that the antibody activity was reduced by 92% (Table 2) and indicate that the epitope specificity of the generated

TABLE 2
Inhibition of glucosylated collagen-reactive antibody by glucitollysine

| ELISA activity* against glucosylated collagen | | |
|---|---|---|
| *Untreated serum* | *Serum pretreated with glucitollysine* | *Percent inhibition* |
| 1.923 ± 0.0118 | 0.162 ± 0.004 | 92% |

*Sera from one rat that was positive to glucosylated collagen were diluted 1:400 and either assayed for activity without further treatment or incubated with 1 mg/ml glucitollysine before assay. Data are expressed as the mean of triplicate samples ± SD.

TABLE 3
ELISA activity of streptozotocin-treated diabetic rats against glucosylated rat skin collagen

| ELISA coating antigen | ELISA* activity of diabetic sera | | |
|---|---|---|---|
| | 1† | 2† | 3† |
| Glucosylated collagen | 0.585 | 0.572 | 0.733 |
| Unmodified collagen | 0.000 | 0.000 | 0.000 |

*Sera from streptozotocin-treated rats with acute diabetes of 5 mo duration were diluted 1:2 and assayed for activity against glucosylated and nonglucosylated rat skin collagen. Data are expressed as the mean of two determinations.
†Animal number.

antibody is directed against the glucitollysine residues of modified collagen.

Supportive evidence that collagen, or other glucosylated proteins, may initiate an antibody response in the diabetic state was obtained in preliminary studies with sera from streptozotocin-treated rats with acute diabetes of 5 mo duration (Table 3). All of the diabetic sera (1:2 dilution) were ELISA-positive against glucosylated rat skin collagen, while none of the sera reacted with unmodified collagen.

## DISCUSSION

In recent years, it has become apparent that increased levels of nonenzymatic glucosylation of several proteins are found in the diabetic state with attendant hyperglycemia.[11–15] This has lead to the postulate that nonenzymatic glucosylation of various proteins may be related to the chronic complications of diabetes.[1] Identification of specific proteins subject to such modification and characterization of the effect of increased glucosylation of these proteins on their structure, function, and immunologic reactivity are essential to the understanding of the relationship between hyperglycemia and long-term complications of diabetes. Collagen, the predominant protein in the body, is the likely candidate for studies involving vascular complications. Not only does collagen comprise a major part of the organic mass of the blood vessels, but it also has a relatively slow turnover rate that could allow for an accumulation of altered protein sufficient for immune stimulation and subsequent immune injury.

Although much controversy still exists regarding the mechanism and factors involved in the pathogenesis of diabetic vascular complications, the role of the immune system appears to be of particular interest. Histopathologic studies have implicated immunologic phenomena in diabetic microangiopathy[16,17] and have shown similarities between diabetic vascular lesions and those found in other diseases with an immunologic pathogenesis.[18] Moreover, diabetic-like vascular lesions can be experimentally induced by immune mechanisms[19–21] and, more interestingly, circulating immune complexes have been described in diabetics with microangiopathy.[22,23]

The data reported in this article further implicate the immune system in the pathogenesis of diabetic complications. We have demonstrated that glucosylated collagen is capable of inducing the production of antibodies with specificity directed against the modified sites in the collagen. In addition, allogenic diabetic sera contain antibodies capable of binding in vitro glucosylated collagen.

Investigation of long-term secondary complications of diabetes mellitus is of great importance, since these complications become, after cardiovascular disease and cancer, a leading cause of death. The possibility that excessive glucosylation and accumulation of altered vascular collagen may be the initiating event of an autoimmune response leading to the vascular complications of diabetes certainly warrants further study.

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