Original Articles

Decreased Collagen Production in Diabetic Rats

ROBERT G. SPANHEIMER, GUILLERMO E. UMPIERREZ, AND VICTORIA STUMPF

Many of the chronic complications of diabetes mellitus involve defects in the connective tissue such as poor wound healing, diminished bone formation, and decreased linear growth. Because collagen is the major protein component of these connective tissues, we examined collagen production in diabetic rats as a probe of this generalized defect in connective tissue metabolism. Doses of streptozocin ranging from 35 to 300 mg/kg were used to induce diabetes of graded metabolic severity in rats. Parietal bone or articular cartilage was removed and incubated at 37°C with 5 μCi L-[5-^{3}H]proline for 2 h, and collagen and noncollagen protein production were quantitated after separation with purified bacterial collagenase. Within 2 wk after induction of diabetes, collagen production was significantly reduced in bone and cartilage from diabetic rats to 52% ($P < .01$) and 51% ($P < .01$) of control (buffer-injected) levels, respectively. In contrast, noncollagen protein production in bone and cartilage from diabetic animals was no different from in tissue from control rats. The correlation between collagen relative to total protein production (relative rate) and the degree of hyperglycemia was highly significant for both bone ($r = -.77$, $P < .001$) and cartilage ($r = -.87$, $P < .001$). Other factors found to correlate with altered collagen production were the duration of diabetes and the amount of weight loss. Thus, diabetes is associated with a marked decrease in collagen production, which was seen early after induction of diabetes and was specific when compared with noncollagen protein production. Cumulative effects of these marked changes in collagen production may contribute to the chronic connective tissue complications in diabetes. *Diabetes* 37:371–76, 1988

From the Department of Medicine, Emory University School of Medicine, Atlanta, Georgia.
Address correspondence and reprint requests to Robert G. Spanheimer, MD, Department of Medicine, Emory University School of Medicine, 69 Butler Street, S.E., Atlanta, GA 30303.
Received for publication 11 February 1987 and accepted in revised form 28 September 1987.

Since the advent of insulin therapy and the resulting marked reduction in acute metabolic complications, the major cause of diabetic morbidity and mortality has been the development of chronic complications. Subsequent observations have revealed that many of these complications are associated with a generalized defect in connective tissue metabolism, including poor wound healing (1), decreased bone mass and rate of formation (2), and thickening of the vascular basement membrane (3). Abnormalities in the connective tissue of one organ may in fact be a signal of more generalized defects, which are seen in patients in whom increased skin thickness was correlated with an increased incidence of retinopathy and neuropathy (4) and in the limited joint mobility syndrome, which was associated with an increased risk of microvascular disease (4,5). Because collagen is the most abundant protein in mammals and the major protein component of connective tissues (6), the widespread nature of connective tissue alterations in diabetes suggests that abnormalities in collagen metabolism may play a role.

Studies of defective collagen metabolism in diabetes have focused on accumulation of collagen over long periods or posttranslational modifications of the collagen peptide rather than the more dynamic changes of collagen synthesis. This has resulted primarily from the availability of techniques to measure these changes, but it has also resulted from the concept that collagen turnover is slow. However, studies of skin fibroblasts from diabetic patients with "thickened-skin syndrome" have demonstrated decreased collagen production in vitro (7), suggesting that defects in the cellular production of collagen may not be reflected in studies of collagen accumulation or posttranslational changes of collagen. In addition, recent studies have demonstrated that collagen production is very sensitive to changes in the nutritional status of animals (8), suggesting closer regulation of collagen production than previously suggested.

To examine collagen production (i.e., synthesis minus

degradation) as a probe of altered connective metabolism in diabetes, we measured collagen and noncollagen protein production in a model that closely resembles protein production in vivo (8), yet over short intervals (2 h) so that accumulation of collagen was not a factor. Results have shown that collagen, but not noncollagen, protein production was significantly decreased in tissue from diabetic animals compared to control animals. Metabolic and nutritional parameters that correlated with this altered collagen production were examined.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (80–100 g) were purchased from Sasco-King (Omaha, NE) and were allowed free access to water and feed (rodent laboratory chow 5001, Ralston Purina, St. Louis, MO) for the duration of the study. Animals were observed for 1 wk in our facility before study. The animal facility was lighted from 0800 to 1800 h, and the ambient temperature was maintained at 24 ± 1°C. For induction of diabetes, animals were placed under ether anesthesia and injected into a lateral tail vein with citrate buffer (0.1 M, pH 4) alone (control) or citrate buffer with 35–300 mg/kg streptozocin (STZ; obtained from the National Cancer Institute, National Institutes of Health). Animals were weighed daily and studied 4–14 days after injection. Animals under ether anesthesia were killed by withdrawal of blood from the abdominal aorta. Blood was centrifuged at 1000× *g* for 10 min at 4°C, and plasma was removed and stored at −20°C for determination of glucose on a Beckman glucose analyzer and for determination of β-hydroxybutyrate (9).

Radiolabeling of tissue in vitro. On study days, animals were killed by ether inhalation. After blood was withdrawn from the aorta, bone was isolated from the parietal aspect of the calvaria, and articular cartilage was isolated from the femoral aspect of the knee. Tissues were cleaned of adherent muscle or tendons and sectioned into halves. Two sections of each tissue from individual animals were incubated for 2 h at 37°C in a shaking water bath with 1 ml modified (10) serum free Eagle's minimal essential media (Sigma, St. Louis, MO) containing 0.5 mM sodium ascorbate (Sigma), 50 μg/ml gentamicin (Sigma), and 5 μCi L-[5-^{3}H]-proline (27 Ci/mmol, Schwarz-Mann, Spring Valley, NY) at a final concentration of 0.1 mM proline. Studies in this laboratory have shown a linear incorporation of [^{3}H]proline into both collagen and noncollagen proteins for up to 4 h after removal of bone and cartilage from the animals.

Collagen and noncollagen protein production. Tissues labeled in vitro were processed with purified bacterial collagenase as previously described for determination of uptake of radioactive proline into collagen and noncollagen proteins (11). Briefly, tissues were minced and homogenized, and then proteins in the combined media and homogenized tissue fraction were precipitated in 10% trichloroacetic acid (TCA) containing 10 mM proline (final concn). After three washes in 5% TCA with 5 mM proline, the final precipitate was dissolved in 0.2 N NaOH at 100°C for 5 min, followed by neutralization for collagenase digestion. Bacterial collagenase was purchased from Cooper (Malvern, PA), and it was further purified by column (1.8 × 131 cm) chromatography via Sephadex G-200 (Pharmacia, Uppsala, Sweden) to remove nonspecific proteases. This purified collagenase was tested for protease activity by incubation with proteins labeled with [^{3}H]tryptophan, obtained from cell culture. Because collagen contains no tryptophan, released [^{3}H]tryptophan after incubation with collagenase indicates breakdown of noncollagen proteins. In addition, *N*-ethylmaleimide (a protease inhibitor) was added so that there was <0.1% degradation of noncollagen proteins after incubation with this protease-free collagenase.

This collagenase technique allowed quantitation of both collagen and noncollagen proteins in the same tissue sample, allowing an independent comparison of collagen and noncollagen protein production. The collagenase-resistant material was hydrolyzed in 6 N HCl at 150°C for 30 min and cooled, and the radioactivity was counted as noncollagen protein. The net incorporation of label into collagen relative to total protein is expressed as the relative rate of collagen production as calculated by the formula

$$\frac{\text{collagen dpm} \times 100}{\text{collagen dpm} + (\text{noncollagen dpm} \times 5.4)}$$

where dpm is disentegrations per minute. Multiplication by 5.4 corrects for the abundance of imino acids in collagen (12).

DNA determination. The DNA content of TCA precipitates (before collagenase digestion) was measured by the diphenylamine method (13).

Statistical analysis. Differences between groups were tested for significance with the unpaired (two-tailed) *t* test. Relationships between variables were tested by linear regression analysis, analysis of variance, or multiple regression analysis.

RESULTS

Variable doses of STZ. Animals were injected intravenously with doses of STZ that varied from 35 to 300 mg/kg to produce diabetes of graded metabolic severity, and they were studied 4–14 days after injection. With 35 mg/kg STZ, plasma glucose increased significantly above that of buffer-injected control animals ($P < .01$), with no alteration in the pattern of subsequent weight gain. With higher doses of STZ,

TABLE 1
Metabolic changes induced by variable doses of streptozocin

Streptozocin (mg/kg)	No. of animals	Plasma glucose (mg/dl)	Weight change (g/day)
0 (control)	35	152 ± 5	5.9 ± 0.3
35	11	258 ± 28*	4.8 ± 0.5
50	33	384 ± 13*	3.6 ± 0.8*
65	11	496 ± 22*	1.9 ± 1.8†
90	8	595 ± 26*	−2.5 ± 0.7*
300	3	613 ± 80*	−6.5 ± 0.5*

Values are means ± SE. Streptozocin in citrate buffer (0.1 M, pH 4) or citrate buffer alone (control) was injected into lateral tail vein while rats were under ether anesthesia. Animals were studied 4–14 days after injection. Plasma glucose was measured on a Beckman glucose analyzer. Differences between streptozocin-injected and control rats were tested for significance with unpaired two-tailed *t* test.
*$P < .01$.
†$P < .05$.

plasma glucose progressively increased until after 300 mg/kg, and then the plasma glucose was >600 mg/dl (Table 1). In addition, with higher doses of STZ, there was a decrease in the amount of weight gain in diabetic animals, until after 65 mg/kg STZ, when equal numbers of animals were gaining and losing weight. There was uniform and significant daily weight loss at a dose >65 mg/kg. Plasma ketones were mildly elevated to 6.9 ± 1.4 mM in animals given 50 mg/kg STZ (vs. 0.16 mM in control, $P < .05$). When all diabetic animals (35–300 mg/kg STZ) were examined as a group ($n = 66$), the mean plasma glucose was 414 ± 52 mg/dl, and the weight change from the time of STZ injection to the time of death averaged 2.3 ± 0.8 g/day, both significantly different ($P < .01$) from control.

Protein production in diabetic and control animals. The effects of 4–14 days of diabetes on bone and cartilage collagen and noncollagen protein production were compared to protein production in age-matched controls. Collagen production was markedly decreased in both bone and cartilage from diabetic compared with control animals (Table 2). In bone from diabetic animals, collagen production was decreased to 52% of control ($P < .01$), with a similar decrease in collagen production to 51% of control ($P < .01$) in cartilage. Thus, in this group of diabetic animals injected with 35–300 mg/kg STZ, collagen production was decreased to one-half of control levels within 2 wk after induction of diabetes in both bone and cartilage.

In contrast, no effect of diabetes on noncollagen protein production was found in either bone or cartilage from the same group of diabetic animals, despite induction of diabetes for up to 2-wk duration (Table 3). In the subset of animals injected with >65 mg/kg STZ, there was a decrease in noncollagen protein production to 70 ± 16% of control levels ($P < .05$). Thus, in diabetic animals with severe metabolic changes and significant weight loss, noncollagen protein production was decreased.

The correlation of plasma glucose (mg/dl) with collagen production relative to total protein production (relative rate of collagen production) in articular cartilage and parietal bone is shown in Figs. 1 and 2. There was a significant inverse correlation between the degree of hyperglycemia and the relative rate of collagen production in both articular cartilage ($r = -.87$, $P < .001$) and parietal bone ($r = -.77$, $P < .001$). Thus, in the two major connective tissue organs of bone and cartilage, the greater the degree of hyperglycemia, the lower the relative rate of collagen production. Because the results are expressed as the relative rate of collagen production in each tissue sample, these changes demonstrate the specificity of diabetes in altering collagen production relative to total protein production at each level of hyperglycemia.

TABLE 2
Collagen production in bone and cartilage from control and diabetic rats

			Diabetic rats	
	n	Control rats (dpm/μg DNA)	dpm/μg DNA	Percent of control
Bone	28	63 ± 8	33 ± 4*	52.4
Cartilage	30	257 ± 22	130 ± 16*	50.6

Values are means ± SE; *n*, no. of separate incubations (2 per animal) for each tissue. Parietal bone and articular cartilage were removed from rats 4–14 days after injection with streptozocin (35–300 mg/kg) in buffer (diabetic) or with buffer alone (control). Tissues were labeled with [^{3}H]proline in vitro for 2 h, and collagen production was determined after purified collagenase digestion of labeled proteins.
*$P < .01$.

TABLE 3
Noncollagen protein production in bone and cartilage from control and diabetic rats

			Diabetic rats	
	n	Control rats (dpm/μg DNA)	dpm/μg DNA	Percent of control
Bone	28	577 ± 63	612 ± 56	106
Cartilage	30	1529 ± 183	1539 ± 105	101

Values are means ± SE; *n*, no. of separate incubations (2 per animal) for each tissue. Noncollagen production was determined after in vitro labeling of tissue from control and diabetic animals after 4–14 days. Statistics with unpaired two-tailed *t* test revealed no significant difference in noncollagen protein production in tissue from control and diabetic animals.

Correlation of articular cartilage protein production with plasma glucose, duration of diabetes, and changes in body weight. Collagen and noncollagen protein production in cartilage from diabetic rats was expressed as a percent of the protein production in cartilage from control rats on each experimental day (control = 100%), and the results were correlated with metabolic and nutritional changes observed in diabetic animals (Table 4). Collagen production was negatively correlated with plasma glucose > duration of diabetes > acute weight change (during 48 h before death) > total weight change (since induction of diabetes). By use of multiple regression analysis, factors were combined and analyzed in pairs for impact on cartilage protein production. Significant ($P < .001$) correlations with collagen production were found for duration of diabetes with total weight change ($r = .742$) > duration with plasma glucose ($r = .732$) > weight change (either 48 h or total) with plasma glucose ($r = .649$). This same order of correlation with the combined factors was found for collagen and the relative rate of collagen production, although in each case the correlation coefficient was higher when the factors were correlated with collagen production.

No single metabolic or nutritional factor correlated significantly with noncollagen protein production in articular cartilage (Table 4). When two factors were analyzed together with multiple regression analysis, only the combined effects of duration of diabetes with total weight change ($r = .268$), and duration with plasma glucose ($r = .25$), were significantly ($P < .05$) correlated with noncollagen protein production.

DNA content. To determine whether diabetes resulted in cellular destruction and altered cellular content of cartilage or bone with the potential for decreased protein production, DNA content of bone and cartilage was measured. As shown in Table 5, total DNA content of diabetic and control bone and cartilage was equivalent.

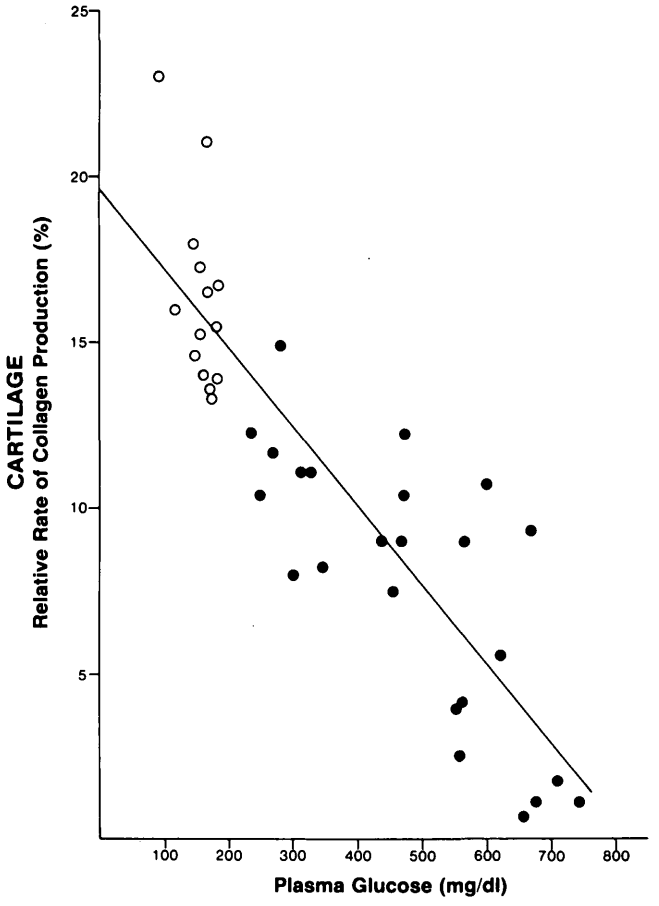

FIG. 1. Correlation of relative rate of collagen production in articular cartilage with plasma glucose. Animals were injected with 35–300 mg/dl streptozocin in buffer (diabetic, ●) or with buffer alone (control, ○). Each *point* represents mean of 2 separate incubations of articular cartilage from 1 animal. Different levels of plasma glucose were achieved in separate animals by injection of variable doses of streptozocin. Tissue was removed 4–14 days after injection, and cartilage was incubated for 2 h with 5 μCi [^{3}H]proline in vitro. Collagen and noncollagen protein production were determined with purified bacterial collagenase, and results are expressed as relative rate as described in MATERIALS AND METHODS. $r = -.87$

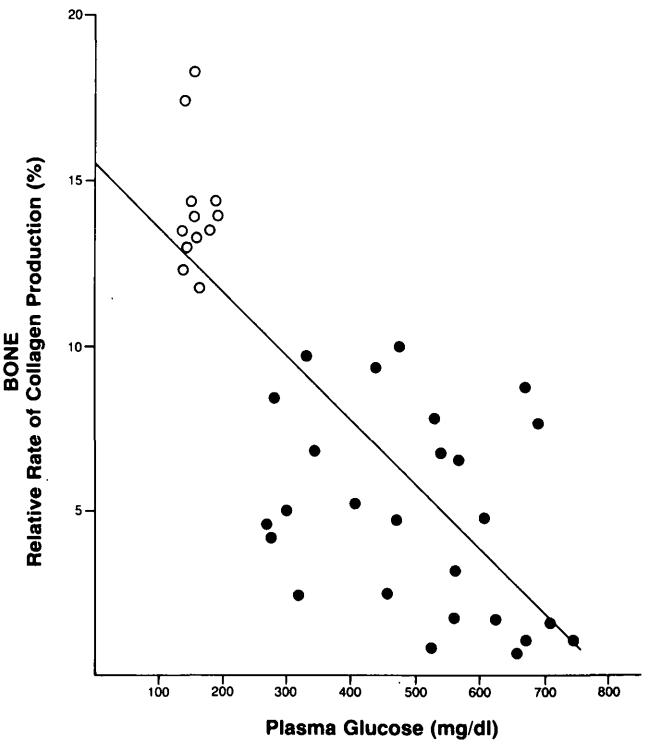

FIG. 2. Correlation of relative rate of collagen production in parietal bone with plasma glucose. Each *point* represents mean of 2 separate incubations of parietal aspect of calvaria bone from 1 animal. ●, Diabetic subjects; ○, controls. Method of treatment and determination of collagen production are same as in Fig. 1. $r = -.77$

DISCUSSION

The magnitude and the specificity of changes in collagen production in diabetic animals demonstrate that collagen is more responsive to the altered conditions of diabetes than was previously suspected. Within 2 wk after onset of diabetes, collagen production was decreased to ~50% of that seen in tissues from control animals. In contrast, noncollagen protein production in the same tissue samples was no different from that in control. However, it could be demonstrated that in a subgroup of severely diabetic animals, noncollagen protein production was reduced to 70% of control levels. Evidence for the specificity of the effect of diabetes on collagen, rather than total protein production,was found when the relative rate of collagen production was compared in tissues from control and diabetic animals over a wide range of plasma glucose levels. With higher plasma glucose levels, there was a lower relative rate of collagen production in both cartilage (Fig. 1) and bone (Fig. 2) so that, at every level of hyperglycemia, collagen was decreased to a greater degree than noncollagen protein production.

Collagen is the most abundant protein in mammals, and distribution of collagen is ubiquitous (6). It provides tensile strength, organization, and integrity to the connective tissues (14); it plays a role in hemostasis through interaction with platelets (15); and it appears to participate in morphogenesis and differentiation during embryonic development (6). The potential implications of the rapid and marked drop in collagen production demonstrated in this study are, therefore, wide in range and may play a role in the chronic complications of diabetes.

TABLE 4
Correlation of articular cartilage protein production with metabolic and nutritional changes in control and diabetic rats

	Correlation (*r*)	
	Collagen	Noncollagen
Plasma glucose (mg/dl)	−.615*	−.184
Days after injection (4–14 days)	−.463*	−.168
Total weight change (g/day)	−.249†	−.072
After 24 h	−.216	.222
After 48 h	−.352†	.070
β-Hydroxybutyrate (mM)	−.095	.041

Collagen and noncollagen protein production in articular cartilage determined as previously described and expressed as percent of buffer-injected (control) animals for each day. Total weight change represents average weight change/day since streptozocin injection, and 24- and 48-h weight changes were obtained before death. Results were tested for significance with unpaired two-tailed *t* test. Number of samples for each variable was 104 (except for β-hydroxybutyrate, $n = 40$) and represents number of separate incubations of articular cartilage (2 per animal).
*$P < .0001$.
†$P < .01$.

TABLE 5
DNA content of bone and cartilage from control and diabetic rats

	Total DNA (μg)	
	Control rats	Diabetic rats
Bone	442 ± 27	449 ± 31
Cartilage	2609 ± 206	2507 ± 183

Values are means ± SE; n = 28 control rats and 35 diabetic rats. DNA content was measured on trichloracetic acid–precipitable fractions of tissue homogenates of entire parietal aspect of calvaria bone or on femoral aspect of knee articular cartilage with diphenylamine reaction. Tissue was removed 4–14 days after injection with 35–300 mg/kg streptozocin in citrate buffer (diabetic) or buffer alone (control). No significant difference was found in DNA content between control and diabetic tissues with unpaired two-tailed t test.

Previous studies confirm our observation of decreased collagen production in diabetes. Diabetic patients have been shown to have poor wound healing (1), and this has been associated with decreased amounts of collagen fibrils (16). In animals injected with radioactive proline in vivo, labeled collagen was found to be decreased by 42% in skin from STZ-induced diabetic rats (17). In this 20-day model, there may have been reincorporation of labeled precursors and accumulation of selected proteins over time, which could be minimized by use of 2-h in vitro incubation, which provides an accurate window on the protein-producing ability of these connective tissues at the time of death of the animal. Thus, the model used in our studies closely reflects collagen and noncollagen protein production in the intact animal (8), but only over short intervals (2 h) designed to reflect collagen production (i.e., synthesis minus degradation) rather than long-term accumulation. It is not known whether the decrease in collagen production observed in our studies was due to a decrease in synthesis or an increase in collagen degradation, but what may be critical to the diabetic patient is a lack of new collagen available for physiologic requirements.

In addition to defects in collagen production, posttranslational modifications of the collagen peptide have also been reported to occur in diabetes. Increased cross-linking of collagen occurs normally with aging, but it is accelerated in diabetic skin and tendon (18,19) and may lead to decreased solubility and increased accumulation of collagen in tissues through an increased half-life. As shown for other proteins, nonenzymatic glycosylation of collagen is also increased in diabetes (20), which may affect collagen metabolism by also changing the half-life of the collagen peptide. It is possible that these posttranslational modifications of collagen in diabetes may lead to excess collagen accumulation under conditions where there is decreased synthesis of collagen (7).

Thus, our studies, in combination with others, suggest the likelihood of more than one defect in collagen metabolism in diabetes: *1*) a decrease in collagen peptide production and *2*) altered posttranslational modifications of collagen that may affect the long-term turnover of tissue collagen.

Diabetes mellitus is a complex disease, characterized by changes in metabolic substrates, hormones, and the general nutritional status. In our studies, we examined the correlation between collagen and noncollagen protein production and altered metabolic and nutritional indices in diabetic animals. We found a significant negative correlation between collagen production and the degree of hyperglycemia, the duration of diabetes, and the amount of weight lost. Higher degrees of correlation were found when two factors were analyzed together for impact on collagen production. When duration of diabetes and the amount of weight loss, or duration and hyperglycemia, were combined, there was a highly significant negative correlation with collagen production ($P < .0001$). Thus, although single metabolic or nutritional factors greatly influence collagen production, it is likely that combinations of these factors may be associated with a much more devastating impact on collagen production in diabetic animals.

The mechanism of altered collagen production in tissues from diabetic animals is unknown, but did not appear to be due to altered cell number as reflected in comparable total tissue levels of DNA from control and diabetic animals. In addition, differences in the specific activity of the cellular proline pools between control and diabetic animals are an unlikely explanation, due to the addition of labeled precursor with high concentrations (0.1 mM) of unlabeled proline.

The generalized nature of abnormal connective tissue metabolism in diabetes suggests that a circulating factor induced by diabetes may play a role in the etiology. Potential circulating mediators of the altered collagen production we observed in diabetic animals include abnormal concentrations of metabolic fuels, hormones, or the presence of inhibitors. Previous in vitro studies have shown that hyperglycemia itself (>20 mM) resulted in an increased collagen production in skin fibroblasts (21) and bovine retinal pericytes (22). In organ cultures of costal cartilage incubated for 24 h in vitro, glucose concentrations <600 mg/dl or ketones (β-hydroxybutyrate) <20 mM did not alter collagen production (23). There is no information on the effects of other metabolic substrates on collagen production.

Potential hormonal mediators of altered collagen production include insulin, which at physiologic concentrations has been shown to increase synthesis of type I collagen in fetal rat calvaria in vitro (24). Glucocorticoid hormone levels are elevated in diabetes, and they have been shown to decrease collagen peptide synthesis in vitro through selective reduction of translatable procollagen mRNA (25). However, the magnitude of decreased collagen production in our studies was greater than that observed due to glucocorticoid excess. In addition, growth hormone levels are elevated in diabetes, but the effects of growth hormone on connective tissues are thought to be mediated through the somatomedins, which have been shown to stimulate bone and cartilage growth, as well as collagen production (26). A discrepancy between a low level of somatomedin on bioassay and a normal level with immunoassay has led to the discovery of a "somatomedin inhibitor" in diabetic serum that has been described by Phillips et al. (27) to inhibit sulfate incorporation into costal cartilage. Recent studies from our laboratory, with a high-molecular-weight fraction of diabetic rat serum that contained the somatomedin inhibitor, demonstrated a marked inhibition of collagen relative to noncollagen protein production in vitro (unpublished observations).

Future studies to determine the mechanism and media-

tor(s) primarily responsible for altered collagen production are critical for understanding the chronic connective tissue defects in diabetes, and they may lead to improved treatment that could be aimed specifically at prevention of these complications.

ACKNOWLEDGMENTS

We gratefully thank L.S. Phillips for helpful discussions and Aaliyah Salim for secretarial assistance.

This work was supported by NIH Grants AM-01435 (R.G.S.) and AM-07298 (G.E.U.).

This study was presented in part at the Southern Section, American Federation for Clinical Research, New Orleans, Louisiana, 1986.

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