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A Study of Connective Tissue Macromolecules in Skin of Mice with Goldthioglucose-induced Obesity (38660)

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An understanding of the effects of obesity on the chemical changes of connective tissue is important, since observations of other phenomena might be misinterpreted when differences in body weight or total composition of tissue are not considered. In an earlier study of skin from rats being treated with insulin, we noted a relationship between animal weight and the content of connective tissue macromolecules (1). Even in inbred strains of animals, growth responses and body weights seemed to affect connective tissue components. In male rats, the heavier and presumably more obese showed relatively lower concentrations of acid mucopolysaccharides (MPS), soluble glycoproteins, and collagen in defatted skin tissue. In contrast, much smaller effects of weight differences were observed in female rats under similar conditions.

Obesity is often associated with hypothalamic dysfunction and has an important relationship to other diseases, e.g., diabetes mellitus. In susceptible mice, goldthioglucose (GTG) has been shown to induce obesity by producing lesions in the ventromedial nuclei of the hypothalamus (2). The present experiment is based on this model which is very useful for studying obesity.

Methods and Materials. One hundred and eighteen male Swiss albino mice (CD-1; Breeding Lab., Charles River, Wilmington, MA) weighing between 18–33 g were used in these studies. Ninety-eight animals were injected intraperitoneally with goldthioglucose (Aurothioglucose, Sigma Chemical Co., St. Louis, MO; 0.5 mg/g body wt), in sterile sesame oil suspension (100 mg/ml), and 20 animals served as controls. All animals were fed freely Purina laboratory chow and tap water. Serial body weights were recorded. Seventeen GTG-treated mice died within 2 wk. At the end of 4 mo, four more had died, and 25 of the surviving 77 GTG-treated mice showed signs of obesity; these were divided into two groups: 12 animals as "moderately obese" (45–56 g) and 13 animals as "obese" (57–80 g). The obese and control animals were killed and skinned; certain organs (heart, spleen, kidney, and liver) were obtained and weighed. All samples were pooled into groups for analysis.

The mice which did not gain weight appeared normal and did not show any signs of toxicity to GTG. Furthermore, since the primary interest in this investigation was to explore the compositional changes in MPS from skin, the toxic effect of GTG, if any, in the animals was not investigated.

Isolation of acid mucopolysaccharides (glycosaminoglycans) (MPS). MPS were extracted from dry, defatted skins with 2% NaOH for 48 hr and then neutralized with 7.35 *N* phosphoric acid. The extracts were digested with pronase (Calbiochem, San Diego, CA, B grade 45,000 PUK units/mg) for 72 hr and were dialyzed simultaneously against 0.1 *M* phosphate buffer, pH 7.8 at 37°. The digested materials were treated with trichloroacetic acid, filtered through celite, and dialyzed against distilled water. The samples were then concentrated, and the total content of MPS isolated from a tissue was estimated by uronic acid analysis (3). Earlier studies indicate the reproducibility of the above method of isolation of MPS (4).

Fractionation of MPS by column chromatography. The isolated MPS mixtures were fractioned on Dowex 1-X2 (AG 200–400 mesh) Cl^- columns, 1.0 × 50 cm by eluting with stepwise-increasing concentrations of NaCl (0.5–4.0 *M*) (5). The effluent fractions were dialyzed, concentrated, and

analyzed for uronic acid. The individual MPS were then characterized and quantitated by analytical procedures reported earlier (6, 7). The Dowex-1 column technique fractionates MPS essentially into groups of compounds, according to their polydispersity and varying degree of sulfation. In general, the fractionation on the Dowex-1 column separates hyaluronic acid (HA) at 0.5 *M* NaCl; chondroitin (Chon), 0.5–0.75 *M* NaCl; heparitin sulfate (HS), 0.75–1.25 *M* NaCl; chondroitin sulfates (CS) A, C, and dermatan sulfate (DS), 1.25–2.0 *M* NaCl; heparin (Hep) and keratan sulfate (KS), 2.0–4.0 *M* NaCl.

Multiple analyses allowed an estimation of an average content of specific MPS in tissue. When large amounts of tissue are not available, as in these studies, gas–liquid chromatographic (GLC) techniques for analyzing uronic acid and hexosamines allow further quantitation of small amounts of MPS. In order to verify the GLC analyses, in previous studies on a number of tissues for which large amounts of material were available, individual MPS were characterized and quantitated by additional purification techniques and by analytical procedures, including uronic acid, hexosamine, acetyl, sulfates, nitrogen, hyaluronidase digestion, optical rotation, chromatographic, and electrophoretic techniques. The results obtained by GLC analyses closely agreed with these results. Fractionation and quantitation of MPS by these procedures gave reproducible results (±15%) and good recoveries of individual MPS (80–90%) (1, 6, 7).

Analysis of MPS fractions by GLC. Fractions obtained from Dowex columns were hydrolyzed in 4 *N* HCl for 16 hr in sealed tubes at 100–105° for hexosamine analyses. Trimethylsilyl (TMS) derivatives were prepared, using hexamethyldisilazane and *N*,*N*-dimethylformamide (7). For uronic acid analysis, samples were hydrolyzed in concentrated formic acid at 100–105° for 20 hr. in sealed tubes under nitrogen, and the TMS derivatives of uronic acid were prepared by the method of Sweeley et al. (8). The conditions of GLC have been described in detail elsewhere (6).

Electrophoresis of MPS. The MPS isolated after fractionation on the Dowex column were also identified by electrophoresis on Titan III cellulose acetate plate, using pyridine-formic acid buffer (50 ml 12 *N* formic acid + 8 ml pyridine + 1150 ml distilled water), 100 V, 20/mA. The MPS were located by staining the strips with alcian blue.

Isolation of glycoproteins. Soluble glycoproteins were isolated from skin which had been dissected free of all extraneous tissue by a method described earlier (9). Briefly, this consisted of extracting minced skin in 15 vol of 0.15 *M* NaCl. The extract was dialyzed exhaustively against distilled water, adjusted to pH 4.0, and fractionally precipitated with $(NH_4)_2SO_4$ at 40, 60, and 100% saturations. The fraction precipitated between 60% and 100% salt saturation contained the soluble glycoproteins. The precipitate was dissolved in water, dialyzed against distilled water, and concentrated by pervaporation.

Analysis of glycoprotein fractions. The isolated glycoproteins were analyzed for protein by a biuret method (10); total carbohydrate by anthrone (11); total hexosamine by the Boas modification of the Elson–Morgan method (12) after hydrolysis in 4N HCl for 14 hr in a sealed tube, omitting the use of resin; and sialic acid by the thiobarbituric acid method of Warren (13).

Studies of collagen and elastin. Different fractions of collagen were extracted by a method described earlier (1) which, briefly, consists of the following steps:

Neutral salt-soluble collagen—Finely minced, wet skin was defatted with acetone for 10 days with six changes of solvent. The defatted skin was dried over H_2SO_4 in vacuo to constant weight and was extracted with 2 vol of 0.2 *M* NaCl buffered with phosphate buffer pH 7.4, $\mu u = 0.02$, 5°. The residue was reextracted three times, the supernatants pooled, and the aliquots hydrolyzed in order to determine hydroxyproline content (14).

Acid-soluble collagen—The residue remaining after the neutral salt extraction was extracted for acid-soluble collagen with citrate buffer, pH 3.5, 0.2 *M*. The extraction

procedure was similar to that used for the salt-soluble collagen.

Insoluble collagen—The residue left after the extraction of acid-soluble collagen was washed several times with distilled water, autoclaved four times at 20 lb/sq in for 15 min, and centrifuged; the supernatant was analyzed for hydroxyproline content.

Elastin—The residue left after autoclaving was washed with distilled water, acetone, and ether, and dried over H_2SO_4 in vacuo to a constant weight. The dried residue after collagen extraction was considered to be elastin.

Results. Although 77 mice received and survived GTG injections, only 25 became obese based on weight curves. The individual differences suggest a marked variability of sensitivity of the hypothalamus to the general toxicity of GTG. Similar differences have been observed among different strains of mice, rats, goats, sheep, and dogs by Baile *et al.* (15). The growth rates of the control, "moderately obese," and "obese" groups of mice are shown in Fig. 1. Although individual variations were noted, there were no appreciable weight differences between obese and control animals until approximately 25 days after injection. The final body weights and the weights of different organs of obese mice are shown in Table I. The actual weights of hearts and kidneys differed little from those of controls, but both spleens and livers were heavier. When related to body weights, the hearts and kidneys were smaller.

Acid mucopolysaccharides. To check the reproducibility of the isolation procedure, skins from control animals were divided into two groups (11 in one and 9 in the other), and MPS were separately extracted and fractioned. The amount of total MPS isolated from the skin of the different groups of mice is shown in Table I. These values are expressed as uronic acid content which represents approximately one third of the parent macromolecule. There was a small

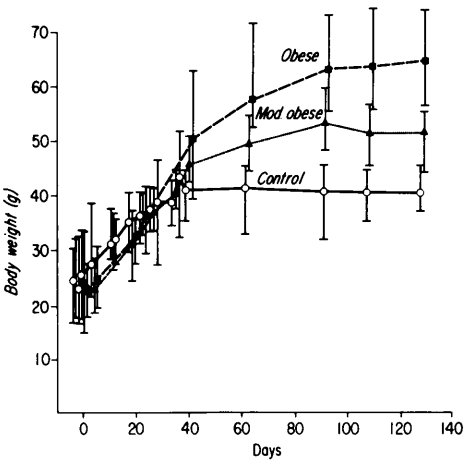

FIG. 1. Body weight time-course of GTG-induced obese mice and controls. Animals were weighed at weekly intervals and the average weight of each group was plotted. Variations within the same group, including controls, were observed. The weight ranges are shown in vertical bars. The difference in body weights between controls and experimental rats became apparent approximately 25 days after GTG injections. (Abbreviation: mod—moderately.)

TABLE I. ORGAN WEIGHTS AND SKIN MPS OF GOLDTHIOGLUCOSE-INDUCED OBESE MICE AND CONTROLS

		Body wt. (g)	Organ weights (g)				Yield of MPS μg/g dry defatted skin[a]
			Heart	Spleen	Kidney	Liver	
Control I	(9)[b]	37.4	0.184	0.073	0.72	2.24	527
		±2.2[c]	±0.022	±0.011	±0.10	±0.40	
Control II	(11)	38.3	0.175	0.070	0.71	2.43	591
		±1.7	±0.021	±0.017	±0.07	±0.33	
Moderately Obese	(12)	51.2	0.162	0.102	0.64	2.45	430
		±3.3[d]	±0.017[d]	±0.040[d]	±0.07[d]	±0.35	
Obese	(13)	65.2	0.185	0.139	0.70	3.52	413
		±5.7[d]	±0.013	±0.029[d]	±0.08	±1.16[d]	

[a] Expressed as uronic acid.
[b] Number of animals.
[c] Standard Deviations.
[d] $P < 0.02$.

difference (12%) between the two control groups, with a mean value of 559 μg uronic acid/g dry skin. Approximately 23% less total MPS content was observed in moderately obese skin, and 26% less in the heavier animals when compared to tissues from controls.

The results of fractionation of crude MPS on the Dowex-1 columns are shown in Table II. Since both the control groups showed almost the same results (differing only by 10%), only the average values are reported. Essentially, there is little difference between the experimental and control groups except that the experimental groups had about 40% lower values of uronic acid in the fraction eluted with 0.5 *M* NaCl when corrected to dry defatted tissue. The GLC analyses and electrophoretic characterization of each fraction are given in the table. In the experimental animals, there was a two- to fivefold increase of galactosamine in the fractions eluted with 0.5 *M* and 0.75–1.25 *M* NaCl. The composition of individual MPS as calculated on the basis of the differential hexosamine and hexuronic acid analysis are shown in Fig. 2.

In general, there is a decrease in MPS containing glucosamine, e.g., hyaluronic acid and heparitin sulfate, and an increase in MPS containing galactosamine, particularly chondroitin. Both tendencies were observed in GTG-treated groups of mice selected as obese.

TABLE II. ANALYSES OF ACID MUCOPOLYSACCHARIDES FROM SKINS OF CONTROLS AND GTG-INDUCED OBESE MICE

Fraction	Gas–Liquid chromatography					
		Hexosamine		Hexuronic acid		
M NaCl	μg Uronic Acid/g dry skin[a]	Glucosamine % of total hexosamine	Galactosamine % of total hexosamine	Glucuronic % of total hexuronic acid	Iduronic % of total hexuronic acid	Electrophoretic identification
Control						
0.50	461	93.3	6.7	100.0	0.0	HA & Chon
0.75	25					HS & Chon
1.00	26[b]	74.9	25.1	73.4	26.6	HS
1.25	19					HS & DS
1.50	40					CS & DS
2.00	14	trace	100.0	48.0	52.0	CS & DS
4.00	6					—[c]
Moderately Obese						
0.50	293	75.7	24.3	100.0	0.0	HA & Chon
0.75	26					HS & Chon
1.00	19	56.5	43.5	89.3	10.7	HS
1.25	18					HS & DS
1.50	37					CS & DS
2.00	34	6.5	93.5	53.5	46.5	CS & DS
4.00	13					—
Obese						
0.50	287	64.6	35.4	100.0	0.0	HA & Chon
0.75	23					HS & Chon
1.00	20	48.8	51.2	75.0	25.0	HS
1.25	19					HS & DS
1.50	37					CS & DS
2.00	20	2.8	97.2	33.3	66.7	CS & DS
4.00	7					

[a] 9.5 mg uronic acid material from control, 4.25 mg from moderately obese and 4.9 mg from obese mice. Skins were fractionated on Dowex-1-column. The recovery from the columns was 101 (89–108) percent.

[b] Samples pooled for quantitative analysis of hexosamine and hexuronic acids.

[c] Not performed due to insufficient quantity.

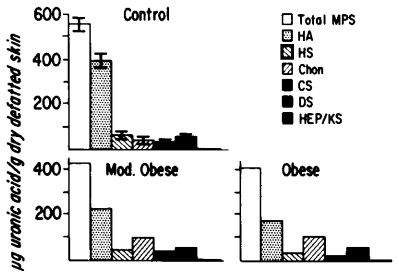

FIG. 2. Composition of acid mucopolysaccharides (MPS) from skin of GTG-induced obese mice and controls. The MPS are expressed as μg uronic acid per g dry, defatted skin. Obese skin had lower contents of total MPS but greater amounts of chondroitin (chon). The control values are the mean of two separate pools of skins; the vertical lines indicate the ranges. (Abbreviations: HA—hyaluronic acid, HS—heparitin sulfate, CS—chondroitin sulfate, DS—dermatan sulfate, HEP—heparin, KS—keratan sulfate.)

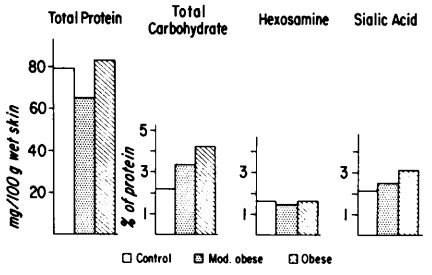

FIG. 3. Carbohydrate composition of glycoproteins from GTG-induced obese mice and controls. The obese mice skin had greater contents of total carbohydrate and sialic acid.

Glycoproteins. Crude glycoprotein was isolated from the skins of two sets of obese mice and from one control group. Analyses of protein and carbohydrate composition for each group is shown in Fig. 3. Although no consistent change in the amount of total soluble glycoprotein was noted, total carbohydrate and sialic acid in the composition of the glycoprotein fraction tended to increase with obesity. Total hexosamine on the other hand tended to remain unchanged.

Collagen and elastin. The collagen and elastin content of obese and control mice skins are shown in Fig. 4. Elastin is expressed in terms of mg dry weight of residue per g dry, defatted skin and was slightly reduced in content in the heaviest animals. Collagen is expressed as mg hydroxyproline

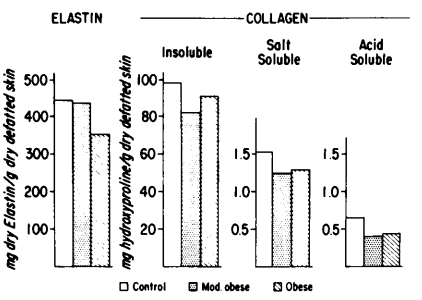

FIG. 4. Elastin and collagen from skin of GTG-induced obese mice and controls. The collagen contents are expressed as mg hydroxproline per g dry, defatted tissue. Note that obese skin had lower content of collagen fractions.

per g dry defatted skin and all collagen fractions, especially the acid soluble material, tended to be less in obese animals than in controls.

Discussion. The association of hypothalamic function with both obesity and diabetes has led many investigators to study the effect of GTG in susceptible animals. GTG-obesity is a model that mimics conditions similar to those observed in other forms of selected hypothalamic injury and, perhaps, to some human diseases. Changes in connective tissue macromolecules are important because they possibly reflect alteration in the metabolism of carbohydrate and protein precursors of these macromolecules, and ultimately such information might contribute to understanding many functional and anatomical alterations throughout the body.

Observations of connective tissue in diabetes mellitus, often accompanying induced obesity, indicate that carbohydrate-protein macromolecular substances play a significant role in this disease process. The excellent chemical studies by Schiller and Dorfman (16) of turnover rates of skin MPS in alloxan diabetic rats demonstrated a decreased metabolism of the MPS which could be restored by administering insulin. Other studies using streptozotocin-induced diabetes in rats likewise revealed a decrease in the content of skin MPS and indicated that there is probably a general reduction of synthesis of all of the MPS, particularly hyaluronic acid and dermatan sulfate (1).

A reversal of such an effect is observed with hyperinsulinism, but in these studies weight gain, even in control animals, was associated with some differences in the content of connective tissue macromolecules. The current studies show a similar decrease in total skin MPS associated with greater weight gain by animals, as noted with hyperinsulinism in a previous study (1). This decrease tends to be reflected mainly by the MPS containing glucosamine and by an increase in chondroitin. The role of glucosamine in obesity is not clear since other studies have shown that glucosamine can inhibit GTG-induced obesity in mice (17). Unfortunately, observations of growth hormone in the present study were not performed. It is known, however, that growth hormone significantly affects connective tissue components by stimulating a secondary serum factor, somatomedin (sulfation factor) (18–21). Sensitivity to growth hormone, although evident in the genetically obese mice, is presumed absent in GTG-induced obesity since no increase in sulfated MPS was observed in these studies but rather a shift toward an increase in chondroitin.

Because of related interest, we also investigated glycoproteins, collagen, and elastin in the skin of mice with chemically induced obesity. The content of the soluble glycoproteins varied. Since the glycoproteins were extracted from wet skin, the fat content might account for some variability; however, the total carbohydrates and sialic acid of the glycoproteins showed a tendency to increase, suggesting an alteration of specific glycoprotein fractions with obesity. Collagen and elastin both showed a tendency to decrease with obesity. Interestingly, similar increases in carbohydrate content of glycoproteins and a decrease in soluble collagen have been noted in the diabetic state (1). It is conceivable that in GTG-induced obesity, a hormonal derangement similar to that in diabetes could be contributing to the changes that are observed at a macromolecular level. These observations emphasize the concept that connective tissue is a target of varied hormonal changes. Furthermore, the data indicated a need to consider body weight when observing compositional changes of connective tissue macromolecules.

Summary. The effect of obesity on the connective tissue composition of skin was investigated in mice with goldthioglucose (GTG)-induced obesity. Four months after GTG treatment, the obese animals were sacrificed. Acid mucopolysaccharides, glycoproteins, collagen, and elastin were analyzed in the skin and compared to the controls. Total MPS in the skin from obese animals decreased, reflected mostly in hyaluronic acid. Chondroitin showed an increase over controls. The content of soluble glycoproteins varied; total carbohydrate and sialic acid of the glycoprotein tended to increase with obesity. Collagen and elastin both tended to decrease with obesity.

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