Studies of Cultured Human Fibroblasts in Diabetes Mellitus

Changes in Heparan Sulfate

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SUMMARY

The incorporation of [^{35}S]sulfate into glycosaminoglycans was studied in cultures of normal and diabetic skin fibroblasts. Heparan sulfate was determined by column chromatography after enzymatic degradation of chondroitin sulfates and dermatan sulfate by chondroitinase ABC. Cultured skin fibroblasts from both insulin-dependent and noninsulin-dependent diabetics were found to have increased proportions of heparan sulfate in the media relative to the other sulfated glycosaminoglycans.
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The role of genetic versus environmental factors in diabetes mellitus has received much attention but remains controversial,[1,2] suggesting the need for new experimental approaches. One method of study that has been applied in other metabolic disorders such as cystic fibrosis,[3] galactosemia,[4] and the mucopolysaccharidoses[5] has been the use of skin fibroblast tissue cultures. We would expect that, if diabetes has a genetic basis, as those other conditions do, it could be similarly studied. To date, skin fibroblast cultures have only been used to a limited degree to study diabetes. One study described a decrease in plating efficiency of "prediabetic" and diabetic fibroblasts.[6] The effect of diabetes on cell growth is unclear. A decreased number of doublings in "prediabetic" and diabetic fibroblasts was reported[7,8]; however, no defects in fibroblast growth were found in insulin-dependent childhood diabetes.[9] A biochemical variation in protein and collagen of diabetic fibroblasts was reported when fibroblasts were grown in media with added hydrocortisone.[8] These findings suggest that the diabetic gene or genes are expressed in the fibroblast and that more definitive biochemical changes or markers might be evident in cultures of diabetic fibroblasts.

Since altered production of the glycosaminoglycan heparan sulfate has been demonstrated in renal cortical tissue in diabetes,[10] this suggested an area for investigation. In our laboratory we have described the structure of heparan sulfate produced by normal skin fibroblasts and the presence of heparan sulfate on the cell surface.[11] We have now extended these studies to examine the production of heparan sulfate by fibroblasts derived from normal subjects, insulin-dependent diabetics, and noninsulin-dependent diabetics.

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MATERIALS AND METHODS

Clinical profile. Skin punch biopsies were obtained from three groups ranging in age from 20 to 62 yr. The normal group consisted of 12 nonobese men with a negative personal and family history for diabetes mellitus including grandparents, aunts, and uncles. Fasting serum glucose and 2-h postprandial serum glucose concentrations were normal. A second group consisted of eight adult, insulin-dependent, nonobese diabetic men who all had a history of ketoacidosis. Age at onset of diabetes was 20 yr or over in all cases. The duration of known diabetes varied from less than 2 yr in three patients to more than 20 yr in five patients. Four patients had no complications of diabetes while proliferative retinopathy, nephropathy, neuropathy, and/or peripheral vascular disease were present in the other four. A third group consisted of five adult-onset diabetic men with no history of ketosis or ketonuria. All were above ideal body weight by 10–20%. Treatment included diet in one, insulin in two, and sulfonylureas in two.

Initiation of cultures. Cell culture products were obtained from Grand Island Biological Company. Punch biopsies of 4 mm were obtained from the lower abdomen. These were cut into small fragments and monolayer cultures established in Eagle's Minimal Essential Medium with the addition of nonessential amino acids, sodium pyruvate (0.11 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. The glucose concentra-

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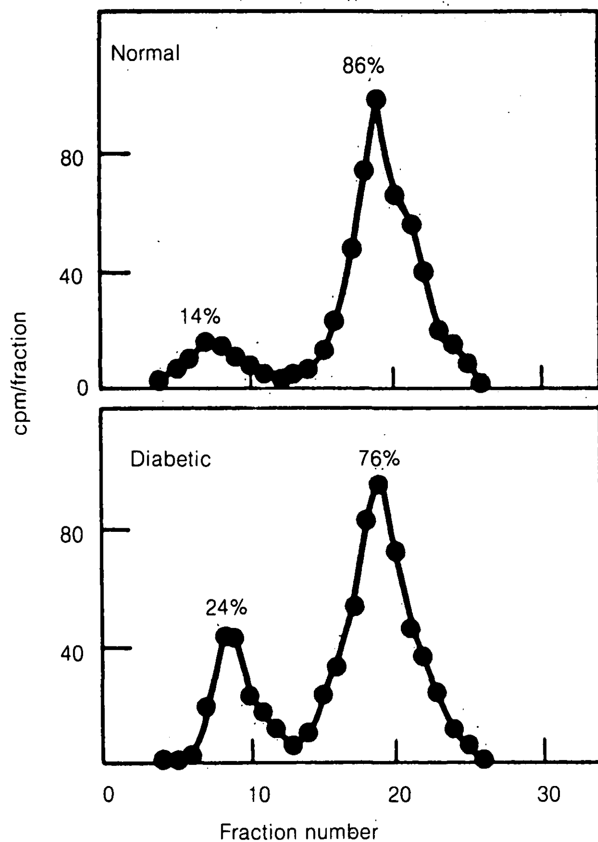

FIGURE 1. Sephadex G-50 column chromatograms of chondroitinase-treated [^{35}S]glycosaminoglycan.

tion of the medium in all studies was 100 mg/dl. Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The medium was changed weekly and the cultures were subcultured upon reaching confluence using a ratio of 1:8.

Experimental technique. After cells had been subcultured one to three times, standard medium was removed and replaced with low sulfate medium containing 0.2 mM inorganic sulfate ($MgCl_2$ replaced $MgSO_4$ to maintain Mg^{++} levels). In addition, the medium contained [^{35}S]sulfate (30 μCi/ml; New England Nuclear) as previously described.[11] Cells were then grown for three days in order to label sulfated glycosaminoglycans uniformly. During this time, skin fibroblasts undergo between one and two doublings and thus will be uniformly labeled. Cells were harvested just before monolayers reached confluence. The media were removed for analysis of glycosaminoglycans. The attached cell monolayer was rinsed six times with 0.15 M NaCl. In several cell cultures cell matrix, cell surface, and intracellular fractions were prepared.[11] A minimum of six culture flasks (Falcon flasks, 25 sq cm) were pooled for each experiment.

Each cell fraction was chromatographed on Sephadex G-25 or G-50[11] to separate [^{35}S]glycosaminoglycan from free [^{35}S]sulfate. Radioactivity was assayed using a Beckman low background planchet counter or a Beckman liquid scintillation spectrometer. The fractions containing the [^{35}S]glycosaminoglycans were pooled and aliquots treated with chondroitinase ABC in order to degrade chondroitin sulfates and dermatan sulfate[12] while leaving heparan sulfate intact. The incubation mixtures contained Chondroitinase ABC (Miles), 0.75 U; chondroitin 4-sulfate (Miles), 0.5 mg; chondroitin 6-sulfate (Miles), 0.5 mg; and enriched Tris buffer,[10] 0.01 ml in a total volume of 0.1 ml. After incubation, the reaction mixtures were chromatographed on a column (1 × 20 cm) of Sephadex G-50 with 0.1 M LiCl as eluant to separate chondroitinase-resistant macromolecular material (i.e. heparan sulfate) from degraded material. Carrier chondroitin sulfates and degradation products were measured using the carbazole method for uronic acid[13] to confirm complete degradation of carrier chondroitin sulfate. The percentage of heparan sulfate relative to the total sulfated glycosaminoglycans was calculated.

RESULTS

Sulfated glycosaminoglycans are the only macromolecular material into which [^{35}S]sulfate is incorporated in cultured skin fibroblasts,[11] and, therefore, [^{35}S]sulfate incorporation is a direct measure of sulfated glycosaminoglycans. Inspection of plates revealed no obvious changes in growth rates of diabetic cells. No appreciable difference in total glycosaminoglycan accumulation was noted between diabetic and normal fibroblasts in media, matrix, cell surface, or intracellular fractions. However this was not examined in detail.

Initially the proportion of heparan sulfate relative to total sulfated glycosaminoglycans was determined in the media,

TABLE 1
Percentage of sulfated glycosaminoglycans in culture media as heparan sulfate

Normal subjects			Insulin-dependent diabetics		
Subject/age		% Heparan sulfate	Subject/age		% Heparan sulfate
P.L.	20	14, 14	E.Q.	24	30, 58†
M.T.	20	11	J.V.	39	52
R.F.	23	17	W.T.	44	30
J.H.	25	20†, 22	J.R.	44	28*
M.T.	27	35†	D.M.	44	35*, 40
J.A.	27	35†	L.F.	52	29
J.C.	41	24†	J.K.	54	60, 71
W.B.	49	25	G.L.	59	37
E.W.	50	18, 20			
J.W.	50	11	Mean		39.8
P.K.	54	16, 19	SD		13.7
O.T.	62	19	P		<0.01
Mean		19.4	Noninsulin-dependent diabetics		
SD		6.6			
Mean ± 2 SD		6.2–32.6	J.G.	41	15
			A.T.	45	52*
			G.V.	50	33
			B.M.	54	35
			R.C.	63	51, 61
			Mean		38.2
			SD		16.5
			P		<0.01

Cell cultures were incubated for 48 h with [^{35}S]sulfate (30 μC/μm). Each analysis is of media pooled from six culture flasks. All studies were at the second subculture except as noted.
* First subculture.
† Third subculture.

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cell surface, and intracellular fractions from two normal and two diabetic cell cultures. Substantial differences between normal and diabetic cultures were seen only in the media. Consequently the other fractions were not investigated in detail or in additional cell lines at this time. Figure 1 shows representative chromatograms of chondroitinase digests of [^{35}S]glycosaminoglycan from the media of a normal and from a diabetic cell culture. In this case the heparan sulfate (undegraded glycosaminoglycan) from the diabetic cell line was 24% of the total sulfated glycosaminoglycan while heparan sulfate from the normal cell line was only 15%. Because of the initial findings of differences in heparan sulfate content, media were examined in detail for heparan sulfate in all remaining cell lines. Results are shown in Table 1. In some cases, more than one experiment was performed with cultured cells from the same patient but at different stages in culture (first to third subculture).

The heparan sulfate of media from normal fibroblast cell lines averaged 19.4% of the total media sulfated glycosaminoglycans. The normal range (mean ± 2 SD) was 6.2–32.6%. Cultures of both groups of diabetic cell lines almost uniformly showed an increased percentage of heparan sulfate in media. The mean values of heparan sulfate in cell lines from ketosis-prone patients and from adult-onset patients were 40.3 and 38.2%, respectively, or about twice the mean value found in media from normal cells. There was no apparent correlation of the proportion of heparan sulfate with the number of passages in tissue culture, the age of the donor, duration of diabetes, or presence of complications. Two fibroblast cultures from insulin-dependent diabetics and one from adult-onset diabetics had values within the normal range.

DISCUSSION

Heparan sulfate has been found in a variety of mammalian tissues[14] including skin,[15] where it is a component of connective tissue ground substance. It is produced in tissue culture by many established cell lines,[16,17] by normal endothelial cells,[18] and by normal human skin fibroblasts,[11] where it is found in both media and in cellular fractions. A number of studies including our own have demonstrated that heparan sulfate is localized to the external cell surface and is the major sulfated glycosaminoglycan on the cell surface.[11,18] It is possible that heparan sulfate may be a component of all cell surfaces.

In the present study an increased percentage of heparan sulfate was found secreted into the medium during the growth phase of fibroblast cultures. This might be likened to in vivo production of altered types of extracellular material. All eight cultures from insulin-dependent patients showed moderate to marked elevations above the mean of the normal group although two of these eight were within the upper limits of the range of normal. One culture from the group of five adult-onset diabetics was not different from normal. This alteration was seen many generations after the cells had been in culture and consequently removed from any effects in vivo such as changes in serum glucose or insulin in the affected diabetic patient. Thus, this finding might represent a genetic difference in diabetes mellitus.

It is not clear if the higher percentage of heparan sulfate is due to increased synthesis or decreased degradation of heparan sulfate. Alternatively, it could be due to decreased synthesis or increased degradation of the other sulfated glycosaminoglycans (chondroitin sulfates and dermatan sulfate). In order to clarify this issue, more detailed studies of all the sulfated glycosaminoglycans in tissue culture will be needed.

The physiologic function of heparan sulfate is not known. At high concentrations, some anticoagulant and lipoprotein lipase-stimulating activity has been reported.[19,20] The presence of heparan sulfate with a highly negative charge on the cell surface makes cell interactions with intercellular connective tissue components a possibility. A recent report that endothelial cell heparan sulfate can be liberated selectively by a platelet-derived enzyme preparation[21] suggests possible endothelial cell-platelet interactions involving modification of cell surface heparan sulfate. Thus there are many possible functional roles of cell surface or intercellular heparan sulfate in both normal and disease states.

It is possible that the accumulation of heparan sulfate in the media of diabetic fibroblasts is similar to altered intercellular glycosaminoglycans and glycoproteins described in the kidney in diabetes.

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