Study of the Hurler Syndrome Using Cell Culture: Definition of the Biochemical Phenotype and the Effect of Ascorbic Acid on the Mutant Cell

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ABSTRACT Fibroblasts from patients with Hurler syndrome retain a distinctive biochemical phenotype when grown in culture which is characterized by increased synthesis of both nonsulfated and sulfated glycosaminoglycans. Ascorbic acid reinforces the phenotypic expression of the biochemical abnormality, producing not only increased synthesis of sulfated glycosaminoglycans, but selective retention of sulfated glycosaminoglycans within the cell. Although the synthesis of nonsulfated glycosaminoglycans is also increased, these compounds, particularly hyaluronic acid are not retained by the cell but are secreted into the medium.

Analyses of urine from patients with Hurler syndrome show increased absolute concentrations of nonsulfated glycosaminoglycans in addition to the expected increase in sulfated glycosaminoglycans. This indicates that the biochemical phenotype as defined in cell culture is not an artifact of the experimental model but reflects the biochemical defect in the patient.

Redefinition of the biochemical defect to include nonsulfated as well as sulfated glycosaminoglycans contradicts explanations of this disease which are based on a single structural gene mutation.

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INTRODUCTION

The Hurler syndrome is a systemic disorder of glycosaminoglycan (mucopolysaccharide) metabolism characterized clinically by skeletal deformities, dwarfism, hepatosplenomegaly, deafness, visual impairment, cardiac abnormalities and mental retardation. These signs result from massive deposition in the tissues of acidic glycosaminoglycans (GAG), particularly dermatan sulfate and heparan sulfate (1). The laboratory diagnosis in affected individuals has been based on the increased urinary excretion of these two compounds (2). Although autosomal and sex-linked modes of inheritance have been recognized, they are as yet biochemically indistinguishable (3).

The lack of a suitable experimental model has restricted the biochemical study of this disease. The clinical features of snorter dwarfism in Hereford cattle suggested a possible animal homologue of the Hurler syndrome (4), but failure to find "gargoyle" cells in the tissues, and excretion in the urine of only chondroitin 4-sulfate make it unlikely that the bovine disease is comparable to Hurler syndrome in man (5).

Several lines of evidence suggested that the use of a human cell culture model might be a productive approach to this problem. Early histologic studies of skin of Hurler patients showed the presence of fibroblasts with metachromatic staining material and "gargoyle" cells (6). Next, metachromatic granules were found to persist for 2–3 wk in cultures of Hurler lymphocytes from peripheral blood (7). More recently, distinctive metachromatic cytoplasmic granules were convincingly demonstrated in serially cultured skin fibroblasts of Hurler patients (8). Finally, it has been shown that serially cultured fibroblasts derived from the skin of normal individuals synthesized a variety of sulfated GAG including dermatan sulfate (9).

The data reported here extend and amplify preliminary studies from this laboratory which demonstrated that the classically accepted biochemical phenotype of Hurler syndrome, i.e., increased dermatan sulfate concentration within cells, persisted in fibroblast cultures derived from the skin of Hurler patients (10). Additional data have been obtained indicating that the biochemical defect is not limited to increased sulfated GAG synthesis but is also characterized by increased synthesis of nonsulfated GAG. The newly synthesized nonsulfated GAG was found in the medium whereas most of the newly synthesized sulfated GAG remained in the cells. Ascorbic acid reinforced the phenotypic expression of the biochemical defects.

These results are consistent with reports since published from two other laboratories which showed increased GAG synthesis in fibroblasts cultured from Hurler patients (11, 12).

METHODS

Cell cultures. Cultures of fibroblasts derived from human skin were used in these experiments. The cells were grown and maintained in Eagle's minimal essential medium 90% (13) with fetal calf serum 10%. This was supplemented with 1 mmole of pyruvate, 0.1 mmole of serine, 10^4 U of penicillin and 500 μg of streptomycin per 100 ml of medium. The methods used for the development of cell lines from biopsy specimens and for cell cultures have been described in a previous report (9).

Experiments were performed on cells which had been serially cultured 6–62 wk. Replicate dishes were prepared containing 2.5×10^5 cells per plate in 10 ml of medium. 5 days after replication 50 μg of ascorbic acid per ml of medium was added to half the plates while the other half served as controls and received no added ascorbic acid. Fetal calf serum contributed 0.01–0.6 μg of ascorbic acid to each ml of medium in all cultures. The medium was replaced three times weekly. Experiments were generally terminated 14 days after the addition of ascorbic acid and the cells were processed for chemical studies.

Chemical methods. The cells were prepared for chemical analyses of GAG by removing the medium and rinsing the cellular layer with Earle's balanced salt solution[1] at 37°C. The rinse was discarded and the cells were removed by scraping into 2 ml of distilled water. Pools of cells were then shell frozen, lyophilized, and weighed. The medium was stored at −6°C until analyzed. GAG were isolated from lyophilized cells and from the medium following the method outlined by Kofoed and Robertson (14). Total sulfated GAG were assayed by a modification of the Rivanol method of Whitehouse and Boström (15), and uronic acid in GAG was measured by the method of Bitter and Muir (16). The total GAG precipitate was fractionated by Svejcar's modification (17) of the procedures originally described by Antonopoulos and coworkers (18). The fractionation of GAG from urine followed the same procedure as for GAG from cells. Results for the cells were expressed as micrograms of GAG by multiplying the uronic acid concentration by 3; this factor reflects the average concentration of uronic acid in GAG. Results of the fractionation of isolated GAG in urine were expressed as milligrams of uronic acid per gram of creatinine. The naphthoresorcinol reaction as described by Pelzer and Staib was used to confirm the presence of dermatan sulfate in fraction 6 and distinguish it from other GAG (19). Hexosamines were assayed by a modification of the method of Cessi and Piliego (20) and their identity established according to Stoffyn and Jeanloz (21). Hyaluronidase digestion of GAG was carried out following the procedure described by Mathews et al. (22). Ascorbic acid was measured by the dinitrophenylhydrazine reaction according to Roe (23).

RESULTS

Cultures of fibroblasts derived from skin of Hurler patients exhibited distinctive metachromatic cytoplasmic granules and "gargoyle cells" when stained with toluidine blue O even after 8 months of serial culture. These observations confirm the data reported on metachromasia in Hurler fibroblasts (8). This staining procedure appeared to be a reliable marker which differentiated Hurler fibroblasts from normal cells.

Glycosaminoglycan concentration in cells. The total cellular GAG concentrations as measured by uronic acid assay, the sulfated GAG concentration, and the effect of ascorbic acid on these components

[1] Prepared from 10 × concentrated stock Earle's balanced salt solution without bicarbonate and phenol red, supplied by Microbiological Associates, Inc., Bethesda, Md.

TABLE I

Cellular Concentrations of Total Glycosaminoglycans (GAG) and Sulfated Glycosaminoglycans (SGAG) in Fibroblasts from Normal Donors and Patients with Hurler Syndrome

Experiment No.	Normal fibroblasts: Cell line	Weeks in culture	GAG: Control	GAG: *Ascorbic	SGAG: Control	SGAG: *Ascorbic	Hurler fibroblasts: Cell line	Weeks in culture	GAG: Control	GAG: *Ascorbic	SGAG: Control	SGAG: *Ascorbic
			μg/mg dry cells						μg/mg dry cells			
1	64–5(a)	33	2.07	1.47	1.23	0.58	65–2(b)	7	7.50	16.17	0.94	6.89
2	65–6(b)	7	16.20	15.30	2.16	3.60	65–5(b)	7	51.30	28.50	3.30	12.70
	65–8(a)	6	19.95	14.70	4.09	12.20	65–7(b)	6	30.30	25.11	5.50	11.00
3	65–9(c)	6	8.67	19.17	1.62	2.28						
4	64–5(a)	62	3.15	2.04	0.84	0.78						
5	65–8(a)	28	14.79	7.80	1.59	1.70	65–5(b)	29	9.42	20.85	1.63	6.67
							65–7(b)	28	27.96	26.31	1.66	5.35
6	66–2(d)	6	3.78	4.86	2.30	3.47	66–1(b)	7	3.51	13.80	2.41	8.00
	66–3(e)	6	13.20	12.18	2.66	4.43						
	Mean		10.23	9.69	2.06	3.63			21.66	21.81	2.57	8.44
	±SEM		± 2.24	± 2.24	±0.30	± 1.48			± 6.18	± 2.19	±0.61	± 1.05
			$P = 0.46$		$P = 0.04$				$P = 0.84$		$P = 0.03$	

Sites from which specimen was obtained: (a) foreskin, (b) biopsy of forearm, (c) surgical specimen from thigh, (d) surgical specimen from supernumerary finger, (e) surgical specimen from back. Specimens 65–7, 65–9, and 66–3 were obtained from female patients. *Each* experiment represents cells cultured at the same time and in the same environment.

* 50 μg of ascorbic acid was added to each milliliter of medium.

are summarized in Table I. The origin of the cells plus a brief history of each cell line is included in the table.

Considerable variability was found in the absolute concentration of cellular GAG per unit mass between individual experiments. This may be a reflection of the biological differences inherent in the patients who were the cell donors, the sites from which the biopsies were obtained, or subtle variations in the conditions under which the cells were cultured. The length of time that the cells were cultured may play a role, as evidenced in both the normal cells (65–8) and the Hurler cells (65–5, 65–7) which showed decreased concentrations of GAG per unit mass in repeated experiments over 28 wk. We were unable to account for the variability, but to control this factor we always studied normal cells at the same time as the Hurler cultures (Table I).

Despite this difficulty, consistent differences were found between the Hurler and normal cells whether one examined the data on the basis of individual experiments or pooled averages. For example, in experiments 2, 5, and 6 the length of time in culture as well as other variables were controlled. Analyses of these experiments on an individual basis showed that addition of ascorbic acid to cultures produced a striking increase in the sulfated GAG concentration of the Hurler cells and a smaller but relatively consistent increase in normal cells. Hurler and normal cells not supplemented with ascorbic acid showed no consistent difference in sulfated GAG concentrations. The results were identical if the pooled averages of all experiments as given in Table I were analyzed. It is of interest that even after 28 wk in culture, the Hurler cells responded to ascorbic acid stimulation, and that ascorbic acid did not alter the total GAG concentration in either the Hurler or normal cells.

Fractionation of cellular glycosaminoglycans. Since the sulfated GAG concentration but not the total GAG concentration was altered by ascorbic acid, its addition to the culture system must have altered the distribution of component compounds measured by the uronic acid assay. This point was confirmed by fractionation of the crude GAG mixture isolated from the cells. The fractionation data from Hurler cells and normal cells are given in Table II. The nominal characterization of compounds eluted in fractions 1–6 was derived from the elution behavior of standard reference compounds. The identity of dermatan sulfate in fraction 6 was confirmed by its resistance to digestion with testicular hyaluronidase and by the fact that a carbazole-naphthoresorcinol ratio of 0.95 was ob-

TABLE II

Glycosaminoglycan Fractionation

Cell line	Total GAG, Fractions 1–6		Fraction 1		Fraction 2		Fractions 3–6		Fraction 6	
	Control	Ascorbic*	Control	Ascorbic*	Control	Ascorbic*	Control	Ascorbic*	Control	Ascorbic*
					μg/mg dry cells					
Normal										
64–5	2.2	2.0	0.7	0.6	0.7	0.5	0.8	0.9	0.0	0.1
	2.9	1.7	2.1	0.7	0.3	0.1	0.5	0.9	0.0	0.0
65–6	20.8	16.9	13.5	6.0	1.7	3.9	5.6	7.0	1.3	1.9
65–8	19.2	18.4	12.0	2.3	1.7	2.7	5.5	13.4	0.9	3.6
65–9	4.5	7.2	1.5	2.0	1.0	2.0	2.0	3.2	0.3	1.0
66–2	2.6	4.1	1.0	0.6	0.5	0.8	1.1	2.7	0.1	0.7
66–3	8.8	10.0	5.1	2.8	1.7	3.1	2.0	4.1	0.2	1.3
Mean	8.71	8.61	5.13	2.14	1.09	1.87	2.50	4.60	0.40	1.24
±SEM	± 2.81	± 2.18	+ 1.89	±0.67	±0.21	± 0.50	±0.75	± 1.58	±0.18	±0.50
Hurler										
65–2‡	8.3	17.9	5.1	3.2	1.8	7.4	1.4	7.3	0.9	2.5
65–5	37.1	29.6	30.8	6.6	4.7	8.9	1.6	14.1	0.6	7.3
65–7	26.8	33.5	16.2	5.5	5.2	12.0	5.4	16.0	2.1	7.2
66–1	19.9	10.0	16.1	2.1	2.2	3.0	1.6	4.9	0.4	3.1
Mean	23.03	22.75	17.05	4.35	3.48	7.83	2.50	10.57	1.00	5.03
±SEM	± 5.24	± 4.67	± 4.57	±0.86	±0.78	± 0.81	±0.84	± 2.30	±0.33	±1.12

Nominal characterization of fractions: 1, low molecular weight GAG, glycoproteins, keratan sulfate; 2, hyaluronic acid; 3, heparan sulfate; 4, chrondroitin 4-sulfate (CSA); 5, chondroitin 6-sulfate (CSC); 6, dermatan sulfate (CSB).

Wilcoxon matched pairs signed ranks test (two sided)

Normal vs. Hurler			
Control		Ascorbic	
1–6	$P = 0.04$	1–6	$P = 0.06$
1	0.03	1	0.12
2	0.01	2	0.02
3–6	NS	3–6	0.04
6	0.14	6	0.02

* 50 μg of ascorbic acid was added to each milliliter of medium.
‡ Fraction 4 lost.

tained. The identity of compounds eluted in other fractions must be interpreted with caution until they are characterized chemically, since net charge, degree of sulfation, and state of polymerization can influence the elution patterns.

The total GAG concentration was obtained by adding fractions 1–6 and the total sulfated GAG by adding fractions 3–6. These data are in reasonably good agreement with the quantitative data obtained by different methods (Table I).

Within the limitations outlined above for the interpretation of the data, obvious differences were found in the distribution of GAG between the Hurler and control cultures. In the absence of added ascorbic acid, the increased concentration of total GAG in the Hurler cells was reflected by the increased concentration of compounds eluted in fractions 1 and 2. Addition of ascorbic acid to the cells decreased the concentration of compounds eluted in fraction 1 and increased the concentration of compounds eluted in fraction 2 and in fractions 3–6. Fractions 3–6 are nominally sulfated GAG and this increase in concentration was similar to the increase found with the Rivanol method (Table I). Dermatan sulfate (fraction 6) was the principal sulfated GAG found in the Hurler fibroblasts.

It was concluded from these data that Hurler cells when cultured in medium containing ascorbic acid contained greater quantities of both nonsulfated and sulfated GAG (particularly dermatan sulfate) than did normal cells.

Net synthesis. The data did not differentiate increased storage of GAG from increased synthesis in Hurler cells. To explore this point, we measured sulfated GAG in the medium and cells and calculated net synthesis. Net synthesis was defined as the sulfated GAG content of the cells plus that of the medium less the sulfated GAG contributed by fetal calf serum. The results of these experiments are summarized in Table III. The data showed net synthesis of sulfated GAG in both the Hurler and normal cultures for the 14 days from day 5 through day 19. Again it was demonstrated that

TABLE III

Net Synthesis of Sulfated Glycosaminoglycans

Line	Days		Normal cells: Cells	Medium	Total	Per cent in cells*	Line	Days		Hurler cells: Cells	Medium	Total	Per cent in cells*
			μg/pool of 10 plates							*μg/pool of 10 plates*			
65–6	5	Control	41	31	72		65–5	5	Control	43	48	91	
	19	Control	70	537	607	5		19	Control	125	735	860	11
	19	Ascorbic‡	327	623	950	32		19	Ascorbic‡	1027	690	1717	60
65–9	5	Control	30	30	60		65–7	5	Control	76	94	170	
	19	Control	107	266	373	24		19	Control	354	647	1001	33
	19	Ascorbic‡	215	350	565	37		19	Ascorbic‡	1260	619	1879	69

* Per cent of newly synthesized sulfated GAG found in cells $= \frac{\text{cells (19 day)} - \text{cells (5 day)}}{\text{total (19 day)} - \text{total (5 day)}} \times 100$.

‡ 50 μg of ascorbic acid was added to each milliliter of medium.

ascorbic acid stimulated sulfated GAG synthesis in the mutant cultures to a greater extent than in the normal cells. When the distribution of sulfated GAG between the cells and medium was examined, it was clear that in ascorbic acid–supplemented Hurler cultures less of the newly synthesized sulfated GAG diffused into the medium than in normal cell cultures.

A different pattern emerged when the synthesis of GAG was determined by uronic acid. Net synthesis of total GAG was several-fold that of sulfated GAG in both cell types, but the Hurler mutant showed greater GAG synthesis. Ascorbic acid increased synthesis of GAG by both cell types; however, the effect on the distribution of total GAG differed from that of the sulfated GAG. Between 70 and 80% of newly synthesized GAG was recovered in the medium of both the normal and

TABLE IV

Net Synthesis of Individual Glycosaminoglycans in 19-Day Cultures

Cell line		Fractions 1	2	3	4	5	6	Total 1–6	Total 3–6
		μg of GAG per pool of 10 plates							
Normal 65–9	Medium*	−91	3521	163	−133	162	310	3932	502
	Cells	96	64	32	22	61	22	297	137
	Total medium + cells	5	3585	195	−111	223	332	4229	639
	Medium + ascorbic acid*	− 1	3961	193	− 3	82	300	4532	572
	Cells	186	193	54	46	107	93	679	300
	Total medium + cells	185	4154	247	43	189	393	5211	872
Hurler 65–2	Medium*	23	1587	308	−229	130	61	1880	270
	Cells	305	110	15	20	15	55	520	105
	Total medium + cells	328	1697	323	−209	145	116	2400	375
	Medium + ascorbic acid*	1160	5332	194	− 2	357	226	7267	775
	Cells	470	1071	235	79	391	366	2612	1071
	Total medium + cells	1630	6403	429	77	748	592	9879	1846
	Control medium‡	91	119	147	343	98	0	798	588

* Net synthesis of GAG in medium.

‡ GAG in equivalent volume (700 ml) of medium before addition to cells. These amounts have been subtracted from the concentration of GAG in medium pools removed from the cultures to obtain net synthesis of GAG.

Hurler cultures, and the distribution of total GAG between the cells and the medium was not altered by ascorbic acid.

Fractionation of GAG from the medium and the cells in a net synthesis experiment is summarized in Table IV and further defines the differences found in the distribution of GAG. The data demonstrated that fraction 2 was the principal GAG secreted by the cells into the medium in both the normal and Hurler cultures. This was characterized as hyaluronic acid after isolation.[2] All of the fractions except fraction 4 (chondroitin 4-sulfate) increased in the medium during 14 days. The increased concentration of compounds eluted in fractions 1 and 2 was most marked in the Hurler cultures supplemented with ascorbic acid.

It was concluded from these experiments that Hurler cells synthesized increased amounts of nonsulfated as well as sulfated GAG and that the increased synthesis of sulfated GAG in the ascorbic acid–supplemented Hurler cultures was associated with an increased retention of newly synthesized sulfated GAG by the cells.

Urinary excretion of glycosaminoglycans compared to cell synthesis. Was the increased synthesis of nonsulfated, as well as sulfated, GAG an artifact of the cell culture system or did it reflect the biochemical defect in affected patients? To examine this question, we fractionated urine specimens from normal individuals, from the four Hurler patients whose cells were studied in culture, and from two additional patients, and compared the results to the distribution of GAG found in the cells. Normal values for urine were based on the distribution of isolated GAG in the urine of 57 healthy individuals aged 0.5–16 yr.[3] Since the concentration of GAG in the urine varies with age, the controls were matched for age with the Hurler patients. Both the Hurler and normal individuals were on unrestricted diets containing ascorbic acid. The urine GAG concentrations were expressed as milligrams of uronic acid per gram of creatinine (Table V) and should be compared to cellular GAG concentrations in ascorbic acid–supplemented cultures (Table II and IV).

TABLE V

Fractionation of Urinary Glycosaminoglycans

	Age	Fraction 1–6	1	2	3–6	6
	yr	*mg of uronic acid per g of creatinine*				
Normal	1	24.5	1.5	1	22	0.5
3 Hurler patients	1	133	15	9	109	45
		283	44	6	233	96
		205	22	30	153	84
Normal	6	9	0.5	0.3	8.2	0.2
1 Hurler patient	6	59	5	5	49	26
Normal	10	6.3	0.4	0.2	5.7	0.2
2 Hurler patients	9	102	7	7	88	48
	10	92	21	1	70	34

Nominal characterization of GAG fractions is the same as that listed in Table II. Normal values are based on the total concentration and distribution of GAG in the urine of 57 healthy individuals age 0.5–16 yr.

The urine from Hurler patients showed increased concentrations of compounds eluted in fraction 1 and hyaluronic acid, in addition to the expected increase in dermatan sulfate. This pattern was similar to that demonstrated in the Hurler cell cultures. The fractionation pattern found in the normal cells was reflected by the fractionation patterns in the urine of healthy individuals.

DISCUSSION

The experiments reported here demonstrate that (*a*) a characteristic biochemical phenotype persists when skin fibroblasts from patients with Hurler syndrome are grown in culture which is characterized by an increased synthesis of both nonsulfated and sulfated GAG, and (*b*) the addition of ascorbic acid (50 μg/ml) to the culture medium produces differential metabolic effects in Hurler cells compared to normal cells as evidenced by increased sulfated GAG synthesis and a selective intercellular retention of sulfated GAG.

The presence of hyaluronic acid has been reported in the Hurler cell but whether this represented abnormal storage with normal synthesis, increased synthesis, or both was not resolved (12). Our data clearly showed increased hyaluronic acid synthesis coupled with secretion of most of the newly synthesized hyaluronic acid into the me-

[2] 102 mg of crude GAG was isolated from 6.5 liters of medium taken from ascorbic acid–supplemented cultures of Hurler cells. The following properties identified it as hyaluronic acid: digestible with testicular hyaluronidase (22); $\alpha_D^{25} - 57.8°$; uronic acid (16) 29.4%; hexosamine (20) 35.6% almost exclusively glucosamine (21).

[3] Van B. Robertson, W., unpublished data.

dium. An increased concentration of nonsulfated compounds eluted in fraction 1 was also present in the medium of Hurler cultures but the significance of this fraction must remain speculative until its chemical composition is further defined.

That the pattern of GAG excretion in the urine of six Hurler patients was similar to that found in the cells suggests that the increased synthesis of nonsulfated GAG found in the Hurler cultures was not an artifact of the cell culture system, and in part validates this system as a model for the disease in man.

Redefinition of the biochemical defect to include nonsulfated as well as sulfated GAG, as suggested by these data, has several interesting theoretic implications. It has been demonstrated that sulfated GAG are covalently bound to protein in the tissues through the OH group of serine (24) and that their synthesis is linked to that of a protein acceptor (25). Two findings point to a primary defect in the linkage of protein to polysaccharide: (*a*) the observation that dermatan sulfate could be extracted with water from the liver and spleen of Hurler patients while similar extractions and purifications of GAG from normal skin required treatment with alkali, or exhaustive proteolytic digestion to separate the polysaccharide from its protein, and (*b*) that dermatan sulfate so isolated from Hurler liver was deficient in serine residues (26). Based on this evidence it has been suggested that the biochemical defect in Hurler syndrome is secondary to a mutation of a single structural gene which results in the synthesis of an abnormal protein that does not effectively bind sulfated GAG (26). Production of a defective binding protein was postulated to produce two effects: (*a*) a deficiency of protein-polysaccharide complex necessary for normal feedback inhibition resulting in excessive sulfated GAG synthesis, and (*b*) diffusion of excess unbound sulfated GAG into the blood stream from which they were deposited in other cells or excreted in the urine.

The data presented in this report are not compatible with this hypothesis in two respects. (*a*) A substantial increase in the concentration of sulfated GAG of the medium would be expected if increased synthesis of GAG were coupled with a defect in binding protein. Although the Hurler cells in these experiments synthesized more sulfated GAG than the normal cells, the excess sulfated GAG remained within the cells and did not increase in concentration in the medium as predicted (Table III). (*b*) In addition, hyaluronic acid synthesis was increased in the Hurler cultures. This is important in view of the increasing body of evidence that hyaluronic acid synthesis may not be linked to the synthesis of a protein acceptor. Hyaluronic acid synthesis is independent of protein synthesis in the streptococcal cell (27). The data is less clear for mammalian cells. In Hurler fibroblasts, puromycin markedly inhibited the incorporation of sulfate and serine into GAG, but had little effect on acetate incorporation (12). Since acetate is a precursor of both hyaluronic acid and sulfated GAG, this differential effect may reflect continued hyaluronic acid synthesis despite puromycin inhibition of protein synthesis.

An hypothesis based on the electronmicrographic demonstration of GAG in hepatic cell lysosomes of Hurler patients suggested that the fundamental defect was related to a deficiency of a normal lysosomal degradative enzyme (28). This concept is in keeping with a mutation of a single structural gene and is supported experimentally by the isolation of partially degraded heparan sulfate and dermatan sulfate from the liver of Hurler patients (29). The present data showing increased synthesis of sulfated and nonsulfated GAG and extensive secretion of newly synthesized hyaluronic acid into the medium by Hurler cells do not support this simple explanation for the disease.

Current knowledge of the intermediary metabolism of GAG does not offer an explanation based on a single structural gene mutation which will account for the phenotypic differences between normal and Hurler cells. Since it is unlikely that several structural genes would undergo mutation simultaneously to reproduce repeatedly the clinical and chemical phenotype of the Hurler syndrome, alternate possibilities must be considered. A mutation in a regulator gene has been considered by several investigators to explain the chemical and clinical characteristics of Hurler syndrome (12, 26). Although the absolute increases in synthesis of several GAG by the cells of Hurler patients and similar increases in GAG excretion in their urine are consistent with this speculation, neither the increased storage of sulfated GAG nor the increased responsiveness of the Hurler cells to ascorbic acid is readily explained on this basis.

The mechanism by which ascorbic acid produces metabolic changes in Hurler cells also remains to be defined. Despite this gap, the relationship established between ascorbic acid and increased synthesis and storage of sulfated GAG may have clinical application if the biochemical controls governing the metabolism of the Hurler fibroblasts in culture are similar to those for fibroblasts in the patient. Studies are now in progress to clarify this point.

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