The Disorder of Hyaluronic Acid Metabolism in Cultured Skin Fibroblasts Derived from a Patient with the Hurler Syndrome

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(Received 21 August 1972)

The metabolism of hyaluronic acid in cultured skin fibroblasts derived from a patient with the Hurler syndrome and from a normal subject was examined. 1. An increased net incorporation of [^3H]glucose into the hyaluronic acid fraction of the Hurler-syndrome cells occurred when compared with normal cells. 2. During a 'chase' period, approx. 35% of the radioactivity derived from glucose was lost from the hyaluronic acid fraction of the Hurler-syndrome cells, whereas the normal cells retained all their radioactivity. 3. Although the Hurler-syndrome cells contained a ninefold greater amount of hyaluronic acid than normal cells, simultaneous determination of the specific radioactivity derived from the label revealed a value for the Hurler-syndrome cells one-half that of normal cells. These results are taken to indicate that the Hurler cells synthesize hyaluronic acid *de novo* at a higher rate than do normal cells. 4. Exposure of Hurler-syndrome cultured fibroblasts to a crude urine corrective-factor preparation (Neufeld & Cantz, 1971), now known to contain α -L-iduronidase, the specific Hurler-syndrome corrective factor (Bach *et al.*, 1972), decreased the hyaluronic acid content to near-normal values before any effect was observed on [^3H]glucose incorporation into the hyaluronic acid fraction. 5. In addition, the hyaluronic acid content of the normal cells decreased after exposure to the corrective factor of urine. 6. The mobilization of hyaluronic acid in Hurler-syndrome and normal cells exposed to the crude corrective-factor preparation of urine caused a decrease in specific radioactivity in the 'corrected' Hurler-syndrome cells and an increase in specific radioactivity in the 'corrected' normal cells.

The demonstration of deficient α -L-iduronidase activity in cultured skin fibroblasts (Matalon *et al.*, 1971; Matalon & Dorfman, 1972) appears to provide a simple explanation for the accumulation of L-iduronic acid-containing sulphated polysaccharides in the Hurler syndrome. However, several aspects of the complex metabolic derangement in the syndrome remain to be clarified. These include: (1) the accumulation in various tissues of neutral glycolipids (Wherret, 1968), gangliosides (Ledeen *et al.*, 1965) and glycopeptides containing mannose and galactose

(Hultberg *et al.*, 1970); (2) deficiencies of specific β -galactosidase isoenzymes in liver and kidney (Ho & O'Brien, 1969), but not in cultured fibroblasts (Fluharty *et al.*, 1970); (3) the accumulation of hyaluronic acid in cultured skin fibroblasts (Matalon & Dorfman, 1966; Schafer *et al.*, 1968), but not in tissues of patients.

The present paper reports our observations on the dysmetabolism of hyaluronic acid in a strain of Hurler-syndrome fibroblasts. It is a by-product of the study described in the preceding paper (Germinario

Table 1. Intracellular glycosaminoglycans of cultured human skin fibroblasts from a Hurler-syndrome patient and a normal subject

The amount of hexosamine present in the hyaluronic acid or sulphated glycosaminoglycan fraction is considered to represent 33% of the total weight of glycosaminoglycan present (Dorfman & Matalon, 1969). Glycosaminoglycan isolation procedures were as described by Germinario *et al.* (1973).

Fibroblasts	Hyaluronic acid ($\mu\text{g}/\text{mg}$ of DNA)	Sulphated glycosaminoglycans ($\mu\text{g}/\text{mg}$ of DNA)
Normal	50.4	11.1
Hurler-syndrome	358.2	352.2

et al., 1973), in which simultaneous labelling with D-[^3H]glucose and [^{35}S]sulphate, and separation of hyaluronic acid from sulphated glycosaminoglycans were carried out to elucidate further the process responsible for storage of extensively degraded sulphated glycosaminoglycans in the Hurler syndrome.

Materials and Methods

The results presented here were derived from experiments described in the preceding paper (Germignano *et al.*, 1973), and the techniques employed were those given in detail there.

Results

The results described in Table 1 indicate that the Hurler strain of cultured fibroblasts had an increased intracellular content of hyaluronic acid (sevenfold greater than normal) as well as of sulphated glycosaminoglycans (35-fold greater than normal). These results confirm those found by others (Matalon & Dorfman, 1966; Schafer *et al.*, 1968). In addition, in the present study, the rate of accumulation of radioactivity in hyaluronic acid of Hurler-syndrome cells

Table 2. Incorporation of [^3H]glucose into the hyaluronic acid fraction of Hurler-syndrome and normal cells

Experiments RG-1–RG-6 represent six separate experiments performed under identical conditions. Experiments showing matched normal and Hurler-syndrome cells were performed simultaneously.

Experiment	[^3H]Glucose incorporated (nmol/4h per mg of DNA)	
	Normal cells	Hurler-syndrome cells
RG-1	1.75* (0.8, 2.7)	47.6* (38.4, 56.8)
RG-2	1.46	6.0* (4.88, 7.15)
RG-3	3.3	10.9
RG-4	3.9	93.9
RG-5	13.0	20.0
RG-6	7.9	32.2

* Averages of values in parentheses.

Fig. 1. Uptake and retention of [^3H]glucose in the hyaluronic acid fraction

(a) Rate of uptake of [^3H]glucose and (b) the retention of radioactivity derived from [^3H]glucose by Hurler-syndrome (●) and normal (○) cultured skin fibroblasts during the 12h 'chase' period with unlabelled medium. The labelling protocol involved a 2h preincubation in unlabelled medium followed by an 8h exposure to double-labelled medium. Each point represents the mean of three experiments.

Fig. 2. Relationship between intracellular hyaluronic acid content and specific radioactivity

(a) Intracellular hyaluronic acid (as hexosamine) of Hurler-syndrome (■) and normal (□) cultured skin fibroblasts and (b) the specific radioactivities (nmol of $[^3\text{H}]$ glucose incorporated/nmol of hexosamine) of the hyaluronic acid fraction in normal (○) and Hurler-syndrome (●) cultured skin fibroblasts after 4 and 8 h incubation in labelled medium. The labelling protocol was as in the legend to Fig. 1. Each point represents the mean of three experiments.

during 4 h exposure to $[^3\text{H}]$ glucose was shown to be greater than that of normal cells, although great variation (1.6–28-fold) occurred among six separate experiments (Table 2). Since all experiments were performed under the same conditions, the cause of this variability is obscure. It could not be explained as a function of age of the cell strain *in vitro* during the various experiments.

In a separate set of experiments (Fig. 1), the uptake of radioactivity was followed by a 'chase' period. The Hurler-syndrome cells incorporated a mean of six

times more radioactivity into their hyaluronic acid fraction than did normal cells, after both 4 and 8 h of exposure to medium containing the label. The clear distinction between Hurler-syndrome and normal cells in the rates of incorporation of $[^3\text{H}]$ glucose into hyaluronic acid, but not by the rates of incorporation of $[^3\text{H}]$ glucose into sulphated glycosaminoglycans (Germinario *et al.*, 1973) is striking. During the 12 h 'chase' period (Fig. 1b), normal cells retained all of their radioactivity, whereas the Hurler-syndrome cells lost 35% of theirs.

The results depicted in Figs. 2(a) and 2(b) have a direct bearing on those described immediately above. Whereas the hyaluronic acid content was eight to nine times greater in mutant than in normal cells after 4 and 8 h exposure to $[^3\text{H}]$ glucose, simultaneous determination of the specific radioactivity derived from the label revealed a value for Hurler-syndrome cells 50% that for normal cells. In combination, the results in Figs. 1 and 2 suggest clearly that the Hurler-syndrome cells synthesize hyaluronic acid *de novo* at a higher rate than normal cells.

Effect of a corrective-factor preparation

In these experiments, the control Hurler-syndrome cells incorporated four to five times more radioactivity from $[^3\text{H}]$ glucose into hyaluronic acid than did normal cells (Fig. 3a). At 4 h after initiation of labelling (18 h after exposure to the corrective-factor preparation), the effect of the preparation was not apparent (Fig. 3a). However, after 8 h in medium containing label and the preparation, the corrective response was striking. The results in Fig. 4 illustrate the contents of hyaluronic acid and the specific radioactivities derived from $[^3\text{H}]$ glucose during the uptake and 'chase' periods of the same experiment described in Fig. 3. When the hyaluronic acid content of the 'corrected' Hurler-syndrome cells decreased to near normal, their incorporation of $[^3\text{H}]$ glucose into hyaluronic acid (Fig. 3a), and its specific radioactivity (Fig. 4c) were, nevertheless, fourfold greater than normal. The observations at 4, 8 and 12 h of the 'chase' period (Fig. 3b) show the influence of the crude corrective-factor preparation on the Hurler-syndrome cells. The increasing specific radioactivity derived from $[^3\text{H}]$ glucose in the 'corrected' normal cells compared with the three other categories is noteworthy.

Discussion

Although an increased content of hyaluronic acid in Hurler-syndrome cells has been observed previously (Matalon & Dorfman, 1966; Schafer *et al.*, 1968), the question whether this was due to deficient degradation, increased synthesis, or both was not resolved. The results reported here provide substantial

Fig. 3. Effects of 'correction' on the uptake and retention of $[^3\text{H}]$ glucose by the hyaluronic acid fraction

(a) Rate of uptake of $[^3\text{H}]$ glucose and (b) the retention of radioactivity derived from $[^3\text{H}]$ glucose during the 12 h 'chase' period with unlabelled medium in normal cells (○), Hurler-syndrome cells (●), normal cells exposed to the corrective-factor preparation (□) and Hurler-syndrome cells exposed to the corrective-factor preparation (■). The 'correction' protocol was as described by Germinario *et al.* (1973). The labelling and 'chase' protocols were as in the legend to Fig. 1.

evidence that cultured Hurler-syndrome skin fibroblasts synthesize hyaluronic acid at a rate greater than normal.

The inability to recognize the Hurler-syndrome cells by $[^3\text{H}]$ glucose incorporation into sulphated glycosaminoglycans contrasts with the ease of differentiating between the two cell types by $[^3\text{H}]$ glucose incorporation into hyaluronic acid. Further, the difference between normal and mutant cells after 4 h labelling is greater in terms of $[^3\text{H}]$ glucose incorporation into hyaluronic acid than of $[^3\text{S}]$ sulphate incorporation into sulphated glycosaminoglycans (Germinario *et al.*, 1973). Indeed, under conditions of pulse-labelling one would expect to observe an aberration in synthesis more clearly than one in degradation. Since little information is available on hyaluronic acid metabolism in the Hurler syndrome *in vivo* (Schafer *et al.*, 1968), the relevance of the finding of increased hyaluronic acid synthesis in Hurler-syndrome cultured skin fibroblasts is unknown. Nevertheless, in the light of these observations, it would be interesting to determine the mechanism for increased accumula-

tion of hyaluronic acid in skin fibroblasts of patients with other mucopolysaccharidoses (Dorfman & Matalon, 1969).

The effects of crude or purified preparations of corrective factor on hyaluronic acid metabolism have not been determined in previous studies (Fratantoni *et al.*, 1969; Neufeld & Cantz, 1971; Barton & Neufeld, 1971). We have now found that when the amount of hyaluronic acid in 'corrected' Hurler-syndrome cells had essentially become normal (Fig. 4a; 18 h after exposure to corrective-factor preparation), their incorporation of $[^3\text{H}]$ glucose into the hyaluronic acid fraction (Fig. 3a), and its specific radioactivity (Fig. 4c) were still fourfold greater than normal. This combination of results indicates that increased synthesis of hyaluronic acid was still occurring at a time when the preparation of corrective factor had led to the elimination of the excess store of hyaluronic acid. After 4 h more of exposure, both indices of $[^3\text{H}]$ glucose incorporation into hyaluronic acid returned towards normal values. The chronology of these events strengthens the view that increased synthesis

Fig. 4. Content of hyaluronic acid (as hexosamine) and its specific radioactivity in Hurler-syndrome and normal fibroblasts with and without 'correction'

(a) Intracellular hyaluronic acid (as hexosamine) during uptake and (b) intracellular hyaluronic acid during the 'chase' period in normal cells (□), Hurler-syndrome cells (■), normal cells exposed to the corrective-factor preparation (▤) and Hurler-syndrome cells exposed to the corrective-factor preparation (▥). (c) Specific radioactivity during uptake and (d) specific radioactivity during the 'chase' period. The symbols in (c) and (d) are as in Fig. 3. The 'correction' protocol was as described by Germinario *et al.* (1973). The labelling and 'chase' protocols were as in the legend to Fig. 1.

de novo is an essential element in hyaluronic acid accumulation by cultured Hurler-syndrome fibroblasts. The absence of hyaluronidase in cultured skin fibroblasts (Matalon *et al.*, 1971) would be a second element contributing to the accumulation of hyaluronic acid.

The progressive increase in specific radioactivity of the 'corrected' normal cells throughout the 12h 'chase' period is notable (Fig. 4d). Such an observation could result from the preferential loss of unlabelled hyaluronic acid alone, or in combination with preferential reutilization of degradation products (containing radioactivity originally present as [³H]glucose) for the synthesis of new molecules of hyaluronic acid. The decreased hyaluronic acid content of 'corrected' normal cells, already apparent 8h after labelling (22h after exposure to corrective factor) favours the former explanation. Thus, both Hurler-syndrome and normal cells mobilized intra-

cellular hyaluronic acid in response to the presence of a crude corrective-factor preparation in the medium, and there is an indication of a difference in their pattern of response. In either case the mobilization was probably caused by increased degradation since little hyaluronic acid was recovered from the medium in this experiment (R. J. Germinario, unpublished work). The apparent decrease in hyaluronic acid between 8 and 12h of the 'chase' period is interpreted to be within experimental error.

In the present study, it could not be determined whether the effects of the corrective-factor preparation on hyaluronic acid metabolism were due to the Hurler-syndrome factor or to impurities in the preparation employed.

This work was supported by grants (MT-3120 and MT-2830) from the Medical Research Council of Canada. R. J. G. is a Lady Davis Institute Post-doctoral Fellow.

The technical assistance of Mr. Gary Miller, Mr. Peter Wechsler, Mr. Peter Halasz and Mr. Fred Baxter is gratefully acknowledged.

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