Variations in Urinary Mucopolysaccharides after Injection of Fibroblasts into Patients with Mucopolysaccharidoses

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Variations in urinary mucopolysaccharides after injection of fibroblasts into two infants with Type I (Hurler's syndrome) or Type II (Hunter's syndrome) mucopolysaccharidosis were followed by daily measurements of total hexuronic acids, sulfaminohexoses, and iduronic acid. For patients being treated with the immunosuppressive agents azathioprine and prednisolone, we observed a significant increase in mucopolysaccharide degradation, but only after a lag time of several weeks. This increase persisted for a very long time while amplifying. When immunosuppressive treatment was omitted or only azathioprine was administered, there was a considerable increase in mucopolysaccharide catabolism during the first 10 days after fibroblast injection. This degradation quickly subsided and was not resumed later. Values for the analytes measured during this early period could be considered as evidence for or against fibroblast rejection. The importance of histocompatibility between donor and recipient and of the administration of immunosuppressive drugs is discussed.

Among the various procedures for correcting the enzyme deficiencies responsible for mucopolysaccharidosis, the injection of fibroblasts from healthy subjects has appeared most attractive, not only because of its simplicity but also because of the accompanying clinical and biochemical improvements reported. The first such trials have shown a persistent return of mucopolysaccharide catabolism towards normal in patients with Type I H, II, or III A mucopolysaccharidoses (1-5). However, almost immediately clinicians have had to confront the problem of survival of the transplanted cells, which are, as all authors agree, markedly immunogenic, even when obtained from histocompatible donors. Rejection of these cells is especially serious because dispersed cells, in general, seem to be particularly sensitive to humoral immunity. For this reason Dean et al. (1-3, 6) suggested treating the patients with immunosuppressive drugs, i.e., by daily administration of azathioprine and prednisolone before and after injection of fibroblasts or of skin-tissue grafting. The usefulness of such a treatment was discussed at length during a symposium in 1979 (7). The question of safety is important because of the hazards to which the treatment exposes the patients.

We became interested in investigating the effect of fibroblast injections into infants with type I H and II mucopolysaccharidoses under various conditions: with standard immunosuppressive treatment (azathioprine plus corticosteroids), treatment with azathioprine alone, and with no immunosuppressive treatment at all. We followed daily the evolution of urinary excretion of acid glycosaminoglycans (GAG) to determine whether differences in the results might provide a criterion for deciding whether, in the absence of focal reaction, the injected fibroblasts have been rejected.

Materials and Methods

Patients and Treatment

Patient 1, a 1½-year-old boy, had Hurler's syndrome (mucopolysaccharidosis Type I), as confirmed by the demonstration of a deficiency in L-iduronidase (EC 3.2.1.76) activity. He received two successive injections of fibroblasts, the first at age 19 months and the second 250 days later, both accompanied by standard immunosuppressive treatment: daily administration of 12.5 mg of azathioprine and 16 mg of prednisolone.

Patient 2, a 4½-year-old boy, had Hunter's syndrome (mucopolysaccharidosis Type II), confirmed by the demonstration of a deficiency in iduronate 2-sulfatase (EC 3.1.6.13) activity. He also received two successive injections of fibroblasts at age 57 and 77 months but under different conditions. The first injection was given without any immunosuppressive treatment; after the second he received 12.5 mg of azathioprine daily with no accompanying corticosteroid.

Fibroblasts

The cell culture was obtained from skin tissue taken from a donor and the fibroblasts were injected according to the procedure of Dean et al. (1). The donor for Patient 1 was his sister (partly HLA compatible) and for Patient 2 his mother (HLA compatible).

Determination of GAG in Urine

After every micturition the urine was immediately frozen and stored at −20 °C. The 24-h urine specimens, reconstituted by thorough mixing of each collection after slow thawing at room temperature, were treated without delay according to a precisely standardized protocol. We purified the polymeric GAG after precipitation with cetylpyridinium chloride, a quaternary ammonium salt. The oligosaccharides

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Nonstandard abbreviations: GAG, glycosaminoglycans; HS, heparan sulfate; DS, dermatan sulfate.
remaining in the supernate were adsorbed onto a 60 × 7 mm column of Dowex 1 × 2 (200–400 mesh, Cl form; Bio-Rad Labs., Richmond, CA). After washing the columns with isotonic saline we eluted the oligosaccharides with a 2.1 mol/L NaCl solution according to Di Ferrante et al. (8). Both fractions were analyzed for (a) total hexuronic acids, measured by the technique of Sajdera as described by Di Ferrante et al. (9), the results being expressed as glucuronic acid; (b) heparan sulfate (HS), measured by the reaction of Lagunoff and Warren (10) and expressed as 2-deoxy-2-sulfaminohepoxide; and (c) dermatan sulfate (DS), estimated by the method of Di Ferrante et al. (11) and expressed as iduronic acid. The procedure is highly reproducible, standard errors for the whole process, from the precipitation step to spectrophotometric measurements, being less than 2%.

Results

Figures 1 and 2 show the results obtained 10 days before and after the fibroblast injection. Marked changes in the excretion of GAG are evident in certain cases.

Total Hexuronic Acids and Sulfaminohepoxides

After immunosuppressive treatment with azathioprine and prednisolone. After two injections of fibroblasts 250 days apart, no notable changes were apparent in the amounts of total hexuronic acids, polymeric GAG, or oligosaccharides eliminated daily or in their composition during the first 10 days after each injection. However, over a longer period of observation, changes became evident that clearly suggested an increase in GAG catabolism (Table 1). The daily mean excretion of total hexuronic acids increased from 21.5 to 32.5 mg for polymeric GAG and from 10.7 to 31.5 mg for oligosaccharides. During that same 250 days the ratio of polymeric GAG/oligosaccharides decreased from 2.01 to 1.03. And it was further decreased to 0.61 by day 500, after the second injection of fibroblasts, after the immunosuppressive treatment had been discontinued. The two fibroblast injections were not followed by any sharp changes in the urinary elimination of GAG in the first 10 days after each injection. On the other hand, eventually there was a progressive increase in GAG catabolism. The data strongly suggest that there was no rejection of the injected fibroblasts in either instance in this patient.

Without immunosuppressive treatment. In contrast, fibroblast injection with no immunosuppressive treatment was immediately followed by a sharp increase of total hexuronic acids and HS in the oligosaccharides for two to three days (Figure 2, IIIa and IIIb). The maximum values were reached the day after the injection, the amounts that day being respectively 2.38 to 2.55 times greater than in the controls (mean 19.5 and 4.1 mg/24 h for the 15 days preceding injection). At the same time, polymeric GAG decreased slightly, so that the ratio for polymeric GAG/oligosaccharides decreased strongly, from 1.96 to 0.66, the day after the injection. However, two days later it increased again to 1.85, almost its previous value. These changes are similar to those observed by Di Ferrante et al. (9) after transfusion of fresh human plasma.

The injected fibroblasts probably immediately released the lysosomal factors acting on the catabolism of the GAG accumulated in the patient's body, a process that seems to imply a rejection phenomenon. In favor of this interpretation is the fact that, contrary to the previous case, there is no change in excretion patterns over a long period. By day 350
(Table 1), the elimination of GAG had decreased and the ratio of polymeric GAG to oligosaccharides was close to its original value, 1.73 vs 1.86 before the injection; moreover, this ratio increased again and was 2.61 by day 600.

We therefore conclude that injection of fibroblasts, even those that are HLA compatible, into a patient with Hunter's disease not treated with immunosuppressive agents is followed immediately by a considerable increase in the elimination of hexuronic acids and of HS in the oligosaccharides. The increase is transient, disappearing in one to five days. Meanwhile, the GAG/oligosaccharides ratio, which considerably decreases during this short period, increases greatly but slowly during the following 600 days—a sign of unfavorable progression in the course of the disease. Despite the absence of focal reaction at the site of the injection, we conclude that a massive and immediate rejection of the injected fibroblasts has occurred.

After immunosuppressive treatment with azathioprine alone. When the patient was treated with azathioprine but with no corticosteroids, the pattern of urinary excretion took still a third course (Figure 2, IVa and IVb). The slight increase, hardly detectable, in total hexuronic acids and of HS of the oligosaccharides on the day of the fibroblast injection is difficult to interpret and we shall discount it. Perhaps it is due to the lysis of a few cells in the preparations injected.

On the other hand, there was a very sharp increase in urinary oligosaccharides on the seventh day after the injection of fibroblasts, which persisted for several days. This was a particularly great increase. The total hexuronic acids of these degraded GAG increased from 11.5 to 54.6 mg in 24 h—i.e., a factor of 4.8—while their HS content increased by 2.7 times. Likewise, the ratio of GAG to oligosaccharides was sharply reduced from 2.65 to 0.48, the GAG decreasing by about 15%. We could only account for these results, as in the previous case, by a sudden mobilization of the GAG deposits due to massive release of lysosomal enzymes, a consequence of rejection of the implanted fibroblasts.

The soundness of this interpretation is strengthened by the fact that the improvement of GAG catabolism was not maintained. Thus in 760 days the ratio GAG/oligosaccharides was 2.47, close to what it was before the fibroblast injection.

Iduronic acid

As was the case for total GAG and HS, the first patient (complete immunosuppressive treatment) showed no increase in urinary excretion of DS shortly after fibroblast injection (Figure 1, Ic and Iic). However, the urinary excretion of polymeric GAG and oligosaccharides of DS origin seemed to increase in the 250-day interval between the two injections (Figure 1, Iic).

The other patient, whether treated with azathioprine alone or with no immunosuppressive treatment at all, also showed no significant increase in the excretion of degradation products of DS during the first 10 days after each injection (Figure 2, IIIc and IVc). We found it rather surprising that in all cases excretion of HS and DS did not follow a parallel course. Whatever the reason, these results lead us to conclude that estimation of iduronic acid does not provide a reliable test for early rejection of the injected fibroblasts.

**Discussion**

Daily measurements of urinary GAG during the 10 days after injection of fibroblasts into a patient with Hunter's syndrome revealed a very marked but transient increase in total hexuronic acids and the sulfaminohexose components of degraded GAG. The observed changes suggest a sudden, albeit ephemeral, increase in GAG degradation mediated by the lysosomal enzymes suddenly released by the transplanted fibroblasts. We ascribe this release to the rejection of the injected fibroblasts, the patient having received either no immunosuppressive treatment or incomplete treatment (azathioprine alone).

In the other case, the patient with Hurler's syndrome received immunosuppressive treatment with azathioprine and prednisolone, and no changes in the urinary GAG were observed during the first 15 days after the injection. However, changes were observed in this patient over a long period, changes that can be interpreted as a progressive resumption of GAG catabolism. Moreover, this catabolism was maintained after the immunosuppressive treatment was stopped. Thus 760 days after the first injection the mean daily excretion of oligosaccharides by this patient had increased by 230% while the ratio of GAG to oligosaccharides had decreased from 2.01 to 0.59. These results lead us to conclude that there was no rejection of the fibroblasts.

We therefore consider it very likely that measuring total hexuronic acids in the urinary oligosaccharides excreted daily during the first 10 days after an injection of fibroblasts should indicate promptly an early rejection of these cells.
that, in the absence of focal reaction, could not otherwise be revealed. Of course, this conclusion must be verified in a greater number of cases.

Furthermore, these results may provide answers to some of the questions raised by administration of fibroblast injections as an enzyme replacement therapy for mucopolysaccharidoses. For example, is complete histocompatibility between donor and recipient indispensable? Our results favor a negative answer. Indeed, two injections of not perfectly compatible fibroblasts were administered without apparent rejection, whereas two injections of HLA-compatible fibroblasts were most probably rejected. The absolute necessity for an immunosuppressive treatment as well as the nature of this treatment is also open to question. Our results support a treatment involving both azathioprine and corticosteroids. Implantation of fibroblasts seems therefore to depend on the maintenance of treatment much as it is currently prescribed. Investigation to determine the minimal doses required does, however, seem necessary.

References

Immunonephelometry of Apolipoprotein B with a Centrifugal Analyzer

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We developed an automated immunonephelometric assay for the measurement of apolipoprotein B (apo B) with a light-scattering microcentrifugal analyzer. Pretreating specimens with a dilute solution of Tween 20 or triglyceride lipase decreased the nephelometric response of apo B. Polyethylene glycol is included in the reaction mixture, and the reaction is complete within 4 min. The method is precise (CV = 6.5%, mean = 0.68 g/L) and the standard curve is linear to an apo B concentration of 2.8 g/L. Lipemia does not interfere with the method if grossly lipemic specimens are centrifuged to remove chylomicrons.

Apolipoprotein (apo) B, the major protein component of very-low and low-density lipoproteins (VLDL and LDL), has received attention because of its use as a risk factor for coronary artery disease (1). Several immunochemical techniques have been used for quantifying apo B, but immuno-

nephelometry is the most suitable for use in a clinical laboratory. Accurate measurement of apo B by immunonephelometry is reported to be difficult because of the heterogeneity of LDL and VLDL and because of nonspecific light-scattering in hyperlipemic serum samples (2, 3). Various methods of pretreatment involving relatively long incubation times have been described to overcome these problems (3–5).

We describe an easy and rapid automated nephelometric method for measuring apo B in both normolipemic and hyperlipemic sera, with no chemical pretreatment of the serum. The remainder of the method is similar to that previously described for the determination of apo A-I (6) and, like the apo A-I assay, has been adapted to a microcentrifugal analyzer capable of measuring scattered light.

Materials and Methods

Apparatus. We used a Multistat® III Fluorescence/Light-Scatter Micro Centrifugal Analyzer (no. 2095) and a Multistat Plus® Loader (no. 840; Instrumentation Laboratory, Spokane, WA 99207).

Reagents. Reagents, suppliers, and concentrations used for the measurement of apo B were as detailed for the assay of apo A-I (6) with two exceptions. Rabbit antiserum specific