Enzyme Replacement Therapy by Transplantation of HLA-Compatible Fibroblasts in Sanfilippo A Syndrome

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Summary

HLA identical fibroblasts were transplanted into three patients with Sanfilippo A syndrome in an attempt to prolong the effectiveness of enzyme replacement therapy. The donors were the patients' mothers or brother and showed no mixed leucocyte reaction when tested against their recipients' cells. Fibroblast cultures from donors were established with 0.2×0.3 cm full-thickness skin biopsies from the anterior surface of the forearm; and their ability to correct abnormal $^{35}\text{SO}_4$ incorporation in the recipients' cells was confirmed before grafting. At the time of grafting, all patients were severely mentally subnormal. All three patients were on an immunosuppressive regimen of 25 mg Imuran and 20 to 25 mg prednisolone daily for 4 wk before fibroblast transplant, and this was continued for approximately 5 months afterwards. When the primary cultures were confluent, the cells were removed by trypsinisation, and approximately 2×10^8 viable cells were injected as a suspension into four subcutaneous dorsal sites.

Excretion of urinary glycosaminoglycans precipitable by 5-aminoacridine increased markedly in all three patients after transplant, the mean increases ranging from 12 to 76%. Uronic acid-containing low-molecular-weight oligosaccharides in the urine also increased considerably in two of the three patients, with mean increases of 3, 65, and 84% being recorded. Oligosaccharides were separated into six fractions (I-Va) by chromatography on Bio-Gel P-2. Urine samples collected before transplant contained a much higher proportion of oligosaccharides of larger hydrodynamic size than did samples taken after treatment. The relative proportions of peaks II-V decreased after transplant, whereas at the same time, there was marked increase in the proportion of peak Va. The sulphamino/hexosamine molar ratios of fraction IV decreased from 0.64 to 0.29 and from 0.53 to 0.35 in two of the three patients after transplant, whereas the third patient showed little change. A protein fraction precipitated by ammonium sulphate from urine samples was tested for its ability to reduce the abnormally high $^{35}\text{SO}_4$ accumulation in fibroblasts cultivated from biopsies taken from each patient before transplant. Most samples taken before transplant had little corrective factor activity, with mean activities ranging from 2.5 to 19% of normal control values. Excretion of corrective factor rose sharply after transplant in all three patients, with a mean level ranging from 48 to 58% of that found in normal age-related controls. The three patients selected for this treatment were observed for periods ranging from 3 years, 3 months to 2 years, 2 months, during which time there were no consistent changes in development achievements, joint mobility, or liver and spleen size as assessed by serial scans.

Speculation

We speculate that the implanted donor fibroblasts survived for a long period of time and possibly even increased in number in the

recipients' subcutaneous space. They released glucosamine *N*-sulphatase, which could enter the patients' cells by adsorptive endocytosis and become incorporated into lysosomes, increasing catabolism of glycosaminoglycan accumulated there. It is possible that repeated fibroblast transplants may increase enzyme levels sufficiently to produce clinical correction, particularly in younger patients in whom physical and mental deterioration has not yet become irreversible.

Sanfilippo type A disease is one of a group of closely related inherited disorders of connective tissue, the mucopolysaccharidoses, which arise from profound deficiencies in one of the several lysosomal exoglycosidase or sulphatase enzymes necessary for complete catabolism of dermatan and heparan sulphates (17). The deficient enzyme in Sanfilippo type A disease is the sulphatase acting on sulphamino groups at the nonreducing termini of partially degraded heparan sulphate chains (15, 24), leading to their massive accumulation as lysosomal storage products and their excretion in large amounts in urine. Somatic changes induced by this accumulation are less marked than in other types of mucopolysaccharidosis, but mental retardation is severe (17, 23). Because lysosomal enzymes can be rapidly taken up by active pinocytosis (10, 13, 20, 22) and become incorporated into phagosomes, together with accumulated storage products, the mucopolysaccharidoses, in common with many other inherited deficiencies in lysosomal enzymes, are well suited to replacement therapy with a suitable source of normal enzymes.

Infusions of normal plasma into three patients with Sanfilippo disease (3) produced specific changes in excreted glycosaminoglycans (GAG) consistent with those expected from increased breakdown and mobilisation of storage products. These changes were not seen when one of these patients was given an equivalent volume of iso-osmotic dextran solution (4). The changes observed after plasma infusion were, however, only transient, presumably because of the short intracellular half-lives of most lysosomal enzymes (10, 19).

The ability of fibroblasts to induce a long-lasting increase in the level of deficient lysosomal enzymes in other types of mucopolysaccharidosis was demonstrated using skin allografts in a patient with Hunter syndrome (5). Moreover, fibroblasts derived from HLA identical donors were transplanted into three further cases of Hunter syndrome and again induced increased catabolism of accumulated GAG, coupled with an increase in activity of the previously deficient lysosomal enzyme iduronate sulphatase (6, 7). We now report the effect of transplanting HLA identical fibroblasts into three patients with Sanfilippo A syndrome in an attempt to prolong the effectiveness of enzyme replacement therapy.

MATERIALS AND METHODS

In all three patients, there was heparan sulphaturia and typical clinical features of Sanfilippo disease (17). The diagnosis of Sanfilippo type A disease was confirmed by demonstration of severe deficiency of heparan *N*-sulphatase activity in cultured fibroblasts. The donors showed no mixed leucocyte reaction against their recipients and were either HLA identical (V. H., K. E.) or had no HLA antigens other than those shared by the recipient (S. B.). Before grafting, the ability of donor fibroblasts to correct abnormal $^{35}\text{SO}_4$ incorporation in the recipient's cells was confirmed in culture (2). At the time of grafting, all patients were severely mentally subnormal. Approval for the transplants was obtained from the Guy's Hospital and Medical School Committee on Ethical Practice, and informed consent for the procedures was obtained from both parents.

V. H.

This girl received her transplant in October, 1976, at age 5 years and 6 months. Developmental retardation was detected at 2 years of age. The patient and her unaffected brother, who was the donor, were both HLA 2, 10; 12, W16.

K. E.

This girl received her transplant in December, 1976, at age 5 years and 2 months. Her parents were first cousins. Developmental retardation was detected at 3 years of age. The patient and her mother, who was the donor, were both HLA 2, 9; 12, 27.

S. B.

This girl received her transplant in November, 1977, at age 12 years and 4 months. Developmental retardation was detected at 3 years of age. The patient was HLA W25, 11; 12, W35 and her mother, who was the donor, was HLA 11; 12, W35.

All three patients were on an immunosuppressive regimen of 25 mg Imuran and 20 to 25 mg prednisolone daily for 4 wk before fibroblast transplant and continued for approximately 5 months afterwards. Patients were assessed before and 6 wk after transplant and at approximately 3-monthly intervals thereafter. In addition to clinical examination, which included photographic recording of joint mobility, the following investigations were made: liver and spleen scans, radiologic skeletal survey, and intellectual and developmental assessments.

LABORATORY PROCEDURES

Urine collections (24 hr when possible) were stored at -20°C with 10 ml of toluene as preservative. Glycosaminoglycans and oligosaccharides were isolated from 100 ml aliquots of each urine collection, as previously described (7). Creatinine was determined by the method of Taussky (21). Uronic acid in urine samples was determined by the method of Bitter and Muir (1) and in column effluents by an automated procedure (11). Hexosamine was estimated as described by Kraan and Muir (14), and sulphamino groups were estimated by the method of Langunoff and Warren (16) after acetylation. Oligosaccharides were fractionated on a column (2.0 $\times$ 100 cm) of Bio-Gel P-2, 200 to 400 mesh (7), and corrective factor was concentrated from urine and assayed as described by Cantz *et al.* (2).

FIBROBLAST CULTURE AND TRANSPLANTATION

Fibroblast cultures from donors were established with 0.2 $\times$ 0.3 cm full-thickness skin biopsies from the anterior surface of the forearm. These were cultured in triplicate in Dulbecco's minimal essential medium supplemented with 4 mM L-glutamine and containing penicillin, 200,000 units, streptomycin, 200 mg/litre, and either fetal bovine serum, 100 ml (for patients K. E. and S. B.) or donor calf serum, 150 ml (for V. H.) per litre, in Falcon tissue culture flasks no. 3024 (Falcon Plastics, Division of BioQuest, Oxnard, CA) in an air incubator at 37°C . When the primary cultures were confluent, the cells were removed by trypsinisation with 0.25% (w/v) trypsin (Wellcome Laboratories, Beckenham, Kent, England) in serum-free phosphate-buffered saline (PBS) and subcultured at a split ratio of 5:1. One further subculture at a split ratio of 6:1 was required to obtain sufficient cells for transplantation. Before transplantation, cells were again trypsinised for 20 min at 37°C in PBS and washed twice in PBS before resuspending them in approximately 4 ml of serum-free culture medium for injection. Approximately 2×10^8 viable cells were injected as a cell suspension into four subcutaneous dorsal sites.

RESULTS

Excretion of urinary glycosaminoglycans precipitable by 5-aminoacridine increased markedly in all three patients after transplant (Table 1). Uronic acid-containing low-molecular-weight oligosaccharides in the urine also increased considerably in two (V. H. and S. B.) of the three patients. Although total uronic acid excretion increased after treatment, the overall levels were far from constant, showing considerable daily fluctuations.

Oligosaccharides were separated into six fractions (I–Va) by chromatography on Bio-Gel P-2, one of which (I) eluted with the void volume and was not analysed further (Fig. 2). Urine samples collected before transplant contained a much higher proportion of oligosaccharides of larger hydrodynamic size (*i.e.*, those eluting towards the void volume) than samples taken after treatment (Fig. 1). When the mean peak areas of fractions II–Va from all samples analysed were calculated and expressed as a percentage of total uronic acid eluted, it could be seen that there was a marked increase in the relative proportions of peak Va, which amounted to 37% in patient V. H., 19% in patient K. E., and 7% in patient S. B.

Fractions V and Va were found to contain less than 2% hexosamine on further analysis, whereas fractions II and III obtained after transplant contained too little total uronic acid for further accurate analyses to be made. Sulphamino-hexosamine ratios were therefore determined only on pooled samples of fraction IV. As can be seen in Table 2, the sulphamino-hexosamine molar ratios of this fraction decreased in two of the three patients after transplant, whereas the third patient (S. B.) showed little change.

A protein fraction precipitated by ammonium sulphate from urine samples was tested for its ability to reduce the abnormally high $^{35}\text{SO}_4$ accumulation in fibroblasts cultivated from biopsies taken from each patient before transplant. The activity of this protein fraction, which in normal individuals contains glucosamine *N*-sulphate sulphatase, the enzyme deficient in Sanfilippo A disease, showed considerable daily fluctuation (Fig. 2). Most samples taken before transplant had little or no corrective factor

Table 1. Mean excretion of uronic acid before and after transplantation

Patient	Glycosaminoglycans (mg uronic acid/g creatinine)			Oligosaccharides (mg uronic acid/g creatinine)		
	Before	After	% change	Before	After	% change
V. H.	80 $\pm$ 45 ¹ (6)	98 $\pm$ 11 (15)	+23	382 $\pm$ 82 (6)	701 $\pm$ 94 (15)	+84
K. E.	137 $\pm$ 13 (8)	154 $\pm$ 10 (25)	+12	632 $\pm$ 48 (8)	648 $\pm$ 54 (34)	+3
S. B.	122 $\pm$ 12 (10)	215 $\pm$ 18 (16)	+76	432 $\pm$ 126 (10)	713 $\pm$ 102 (14)	+65

¹ Mean $\pm$ S.E. from the numbers of analyses (shown in parentheses).

Fig. 1. Elution profiles on Bio-Gel P-2 of oligosaccharides isolated from urines of controls and patients V. H., K. E., and S. B. at days before (negative numbers) and after (positive numbers) transplant.

activity, with mean activities ranging from 1.8% of normal control values in the case of patient V. H. to 8.0% with patient S. B. (Table 3). Excretion of corrective factor rose sharply after transplant in all three patients, with a mean level ranging from 48 to 58% of that found in normal age-related controls.

The three patients selected for this treatment were all severely affected both physically and mentally. They were observed for periods ranging from 3 years, 3 months to 2 years, 2 months, during which time there were no consistent changes in developmental achievements, joint mobility, or liver and spleen size as assessed by serial scans.

DISCUSSION

The biochemical changes observed here after transplantation of HLA identical fibroblasts were remarkably similar to those previously seen when three patients with Hunter syndrome (α -L-idurono-2-sulphate sulphotase deficiency) received fibroblast transplants (7). These changes included an increased excretion both of GAGs and oligosaccharides of lower molecular weight. A decrease in the relative proportions of the larger oligosaccharides

Table 2. Sulphamino/hexosamine molar ratios of oligosaccharide fraction IV separated on Bio-Gel P.2¹

Patient	Before	After
V. H.	0.64	0.29
K. E.	0.53	0.35
S. B.	0.44	0.50

¹ Four samples were pooled in each group from urines collected 0 to 5 months before and 1 to 11 months after transplant.

separated by chromatography on Bio-Gel P-2 was accompanied by a corresponding increase in the oligosaccharides of lowest hydrodynamic size. Further, there was a decrease in the sulphamino content of one of the major oligosaccharide fractions in two of the Sanfilippo patients, which was similar to the decrease in sulphate content of an equivalent fraction previously observed in all three treated Hunter patients (7).

In patients with a deficiency of specific lysosomal sulphatase enzymes, a measurable decrease in the sulphate content of urinary oligosaccharides after fibroblast transplant is highly indicative of

Fig. 2. Urinary output of Sanfilippo A corrective factor in three patients with Sanfilippo A syndrome. Negative numbers, samples taken before transplant.

Table 3. Mean excretion of Sanfilippo A corrective factor before and after transplantation

Patient	Before	After
V. H.	$3.2 \pm 31^1 (3)^2$	$100 \pm 30 (12)$
K. E.	$15 \pm 8 (4)$	$86 \pm 25 (15)$
S. B.	$4.5 \pm 3 (3)$	$104 \pm 20 (11)$
Control	$178 \pm 31 (3)$	

¹ Mean units of correction $\pm$ S.E. as defined by Barton and Neufeld (1971) per g creatinine.

² Numbers in parentheses, numbers of samples analyzed.

increase in activity of the deficient enzyme. This is further evidenced in the present cases by the accompanying increase in the total urinary excretion of oligosaccharides, and by a decrease in the proportion of higher molecular weight oligosaccharides. These effects are compatible with catabolism of stored GAGs.

More direct evidence for increase in the activity of glucosamine *N*-sulphate sulphatase in all three patients after treatment was provided by large increases in the activity of urinary "corrective factor," which contains this enzyme. We measured specific correc-

tive factor rather than urinary or leucocyte glucosamine *N*-sulphatase because the former assay is more sensitive. Thus, duplicate assays for heparan sulphamidase (glucosamine *N*-sulphate) in normal leucocytes varied by 10 to 15% so that small increases in the activity in patients' leucocytes would not be detected. In Sanfilippo type B cells cultured in medium with added α -*N*-acetyl glucosaminidase, for example (19), increases of only 2 to 5% induced a 42 to 70% correction of radiolysulphate metabolism.

Both the increase in catabolism of accumulated storage products and the increase in the activity of corrective factors we observed here persisted after immunosuppressive therapy had ceased.

Fibroblasts were chosen as a source of lysosomal enzymes because they appear to have the capacity to secrete many of the enzymes deficient in each type of mucopolysaccharidosis (10, 18, 19), obviating the need for prior enzyme purification and the attendant dangers of altering enzyme recognition markers or immunologic status. Normal skin fibroblasts can correct the abnormal ³⁵S-sulphate incorporation of fibroblasts from patients with mucopolysaccharidosis when grown together in culture (9) because they appear to release lysosomal enzymes which can be taken up by deficient fibroblasts and so restore normal physiologic functions (9). A good histocompatibility match between patients and donors, together with immunosuppressive therapy will promote long-term survival of donor fibroblasts. Exchange of lysosomal enzymes *in vivo* from normal to deficient cells has been shown to take place between leucocytes in cattle (12) and between hepatocytes in mice (8). Increases in catabolism of GAG and excretion of corrective factor demonstrated here over a long period indicates that fibroblast transplantation has promise as a method of enzyme replacement therapy. However, the observed biochemical changes appeared rather too large to be accounted for by the relatively small numbers of cells transplanted, particularly because we have found that fibroblasts release only a small proportion of their lysosomal enzymes into their culture medium under normal *in vitro* conditions. Nevertheless, this small amount of enzyme may be sufficient to restore normal physiologic functions to a considerable degree. Alternatively, the donor cells may divide *in vivo* and increase in number or may be stimulated to release much larger amounts of lysosomal enzymes than are normally produced.

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