Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 7472–7474, October 1986
Medical Sciences

Glucocerebrosidase processing in normal fibroblasts and in fibroblasts from patients with type I, type II, and type III Gaucher disease

(pulse label/diagnosis)

ERNEST BEUTLER* AND WANDA KUHL

Scripps Clinic and Research Foundation, Department of Basic and Clinical Research, 10666 North Torrey Pines Road, La Jolla, CA 92037

Contributed by Ernest Beutler, June 23, 1986

ABSTRACT **Fibroblasts from normal subjects and patients with the three types of Gaucher disease were labeled with [^{3}H]leucine. Glucocerebrosidase antigen was immunoprecipitated using affinity-purified Sepharose-bound antibody. Normal cells initially formed a 60-kDa polypeptide antigen that was gradually replaced by a broad band of antigen averaging 63 kDa. This position corresponds with that of mature fibroblast and placental enzyme. Processing of glucocerebrosidase in six unrelated patients with type I Gaucher disease and one patient with type III Gaucher disease was exactly the same as normal. In contrast, three patients with the severe infantile (type II) form of the disease manifested a very unstable enzyme; the 60-kDa band appeared transiently and the mature 63-kDa band was never seen. These results indicate that type II Gaucher disease may well be distinguishable from type I disease by virtue of the very unstable enzyme precursor. Contrary to some earlier reports, processing of glucocerebrosidase in type I disease appears to be entirely normal.**

Gaucher disease is characterized by a deficiency in the activity of the enzyme glucocerebrosidase. In patients with the most common form of the disease, designated type I or "adult," the reticuloendothelial system but not the central nervous system is involved. In contrast, neurologic involvement occurs in the rare type II, or "infantile," and type III or "juvenile" forms of Gaucher disease. Complementation studies indicate these disease forms are due to mutations involving the same gene (1, 2), but the reason for the very different disease phenotypes is, as yet, unknown.

Ginns *et al.* (3) carried out electrophoretic transfer blots on fibroblast extracts from patients with the different types of Gaucher disease using rabbit antisera and suggested that type I disease might be due to a processing error. In our own studies, using affinity-purified heterosera and monoclonal antibodies, we found only a 63-kDa antigen in normal fibroblasts and fibroblasts from patients with type I and type III Gaucher disease. No band was visualized in type II disease. We suggested that the other bands that had been observed by Ginns *et al.* represented antigens not related to glucocerebrosidase.

We now present the results of pulse–chase experiments demonstrating that glucocerebrosidase processing does occur and that processing is quite normal in six patients with type I and one patient with type III Gaucher disease. However, an unstable precursor is formed in type II disease.

MATERIALS AND METHODS

Cultures. Confluent skin fibroblasts cultured in minimal essential medium (MEM) with 20% fetal bovine serum were used in these investigations. Cell lines GM372, GM1260, and GM877 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Other cell lines were derived from our patients with Gaucher disease. Of the type I patients studied, five are Jewish; one is non-Jewish.

Antibodies. The production of affinity-purified anti-glucocerebrosidase antibodies in rabbits has been described (4). Antibody and preimmune IgG each were immobilized on cyanogen bromide-activated Sepharose beads (1 mg/g of Sepharose; Pharmacia) using the technique recommended by the manufacturer.

Pulse Labeling. To pulse label the cells, supernatant culture medium was removed from confluent cultured fibroblasts, they were rinsed with saline, and the medium was replaced by leucine-free MEM containing 5% fetal bovine serum and 0.5 mCi of [^{3}H]leucine (120–190 Ci/mmol; 1 Ci = 37 GBq; Amersham). After 30 min the medium was removed and the cells were washed four times with saline followed by addition of MEM with 20% fetal bovine serum. Penicillin and streptomycin were used in all medium preparations. One or two flasks containing cultured fibroblasts were sacrificed immediately after labeling (time = 0), at 2 hr, and at 24 hr. The cells were again washed with saline and removed from the flask into saline with a rubber policeman. The cells were pelleted by centrifugation, dissolved in 0.2–0.3 ml of Tris/NaDodSO$_4$ (final concentration, 0.03 M Tris, pH 6.8/1.5% NaDodSO$_4$), and briefly sonicated. The preparations were placed in boiling water for 3 min and frozen until all time points could be processed together.

Immunoprecipitation. Glucocerebrosidase antigen was immunoprecipitated using Sepharose-bound antibody. The thawed cell extract was centrifuged at 12,000 × *g* for 3 min and diluted 1:10 in 0.05 M Tris (pH 6.8) containing 0.1% Triton X-100, 0.4 M NaCl, and 0.6% ovalbumin. Before treating the cell extract with anti-glucocerebrosidase antibody, it was preabsorbed with beads prepared from preimmune rabbit IgG. Two hundred microliters of beads was added to 2 ml of diluted cell extract and placed on a tube rotator for 1–2 hr at 22°C. The preabsorbed extract was removed from the beads and was absorbed with anti-glucocerebrosidase beads in the same manner. The beads were transferred to a minicolumn and were washed seven times with 1-ml portions of 0.05 M Tris (pH 6.8) containing 0.4 M NaCl, 0.1% Triton X-100, and 0.6% ovalbumin and then three times with 1-ml portions of the same mixture with the pH adjusted to 8.2. Finally, the beads were rinsed from the column with 0.05 M Tris (pH 6.8) and allowed to sediment. After removal of the supernatant fluid the bound antigen was removed from the beads by placing in a boiling bath for 3 min with an equal volume of 0.06 M Tris (pH 6.8) containing 3% NaDodSO$_4$, 50 mM dithiothreitol, and 10% glycerol.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed.

Controls. In the case of the 0-hr and 24-hr samples, the cell extract from two flasks was pooled and preabsorbed with the preimmune IgG beads. Then one-half of the extract was treated a second time with preimmune IgG beads while the other half was treated with the anti-glucocerebrosidase beads. After washing as described above, the bound material was removed from the beads with $NaDodSO_4$ and boiling.

Electrophoresis. Electrophoresis of immunoprecipitated extracts was carried out according to Laemmli (5) in 10% acrylamide gels (length, 12 cm) with 5% stacking gels. The electrophoresis was continued until 30 min after the bromophenol blue had migrated off the gel. The gel was fixed overnight in 40% methanol with 10% acetic acid, soaked in EN^{3}HANCE (New England Nuclear, Boston, MA), and dried on a gel dryer. XAR film (Eastman, Rochester, NY) was exposed to the dried gel for 7–28 days at −70°C.

RESULTS

Figs. 1–3 are fluorograms of the labeled, immunoprecipitated proteins of fibroblasts from normal individuals and patients with type I, type II, and type III Gaucher disease. The major bands of radioactivity were at 56, 60, and 62–64 kDa. For the sake of convenience the latter is referred to as the 63-kDa band.

In interpreting the results of these studies it is essential to recognize that the 56-kDa band was also present when the extract was treated with preimmune rabbit serum beads (control channel). Thus, this band cannot be considered to represent glucocerebrosidase antigen; rather, it is a labeled protein that is avidly bound by rabbit immunoglobulin. In addition to the major 56-, 60-, and 63-kDa bands, bands are seen at about 100 kDa, which we believe represent low-affinity, immunocross-reactivity with β-galactosidase (4). Several weakly radioactive bands were also present, but since these minor bands were equally represented in the control channels they are not considered to represent glucocerebrosidase antigen.

The first glucocerebrosidase band to appear in the normal Gaucher disease fibroblasts was the 60-kDa polypeptide. In the normal, type I, and type III Gaucher disease fibroblasts this was then converted to the 63-kDa polypeptide, which then gradually disappeared. In the case of the type II Gaucher disease fibroblasts the initial 60-kDa band was seen at 0 and sometimes at 2 hr but it was quite evanescent. No 63-kDa band was formed.

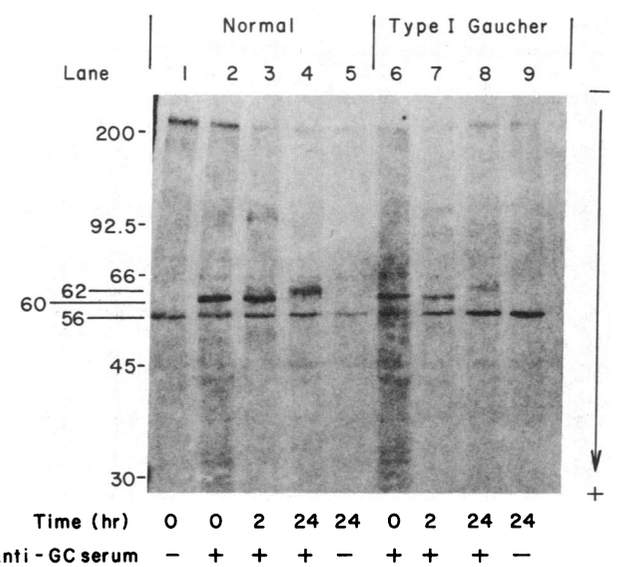

FIG. 1. $NaDodSO_4$ electrophoresis of immunoprecipitated fibroblast extracts from normal (lanes 1–5) and type I Gaucher disease (lanes 6–9). Extracts were prepared at completion of pulse (time = 0) and after 2 and 24 hr of chase (time = 2, time = 24). At times 0 and 24 one-half of the extract was treated with anti-glucocerebrosidase (Anti-GC) beads (+) and as a control the other one-half was treated with preimmune IgG beads (−). Sizes are given in kDa.

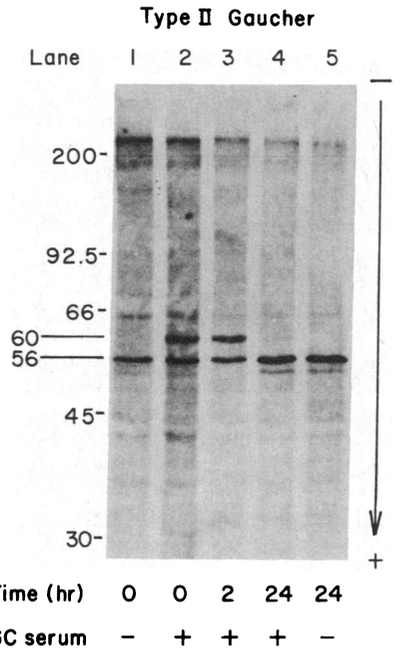

FIG. 2. $NaDodSO_4$ electrophoresis of immunoprecipitated extracts from type II Gaucher disease fibroblasts. See Fig. 1 legend for details.

DISCUSSION

The primary product of glucocerebrosidase mRNA is a peptide with a molecular mass of either 57.5 or 60 kDa. The actual size depends upon which of two possible 5′ methionine sites is used to initiate translation. The primary polypeptide product is known to be cleaved to a mature enzyme, either 19 or 39 amino acids being removed in the course of processing

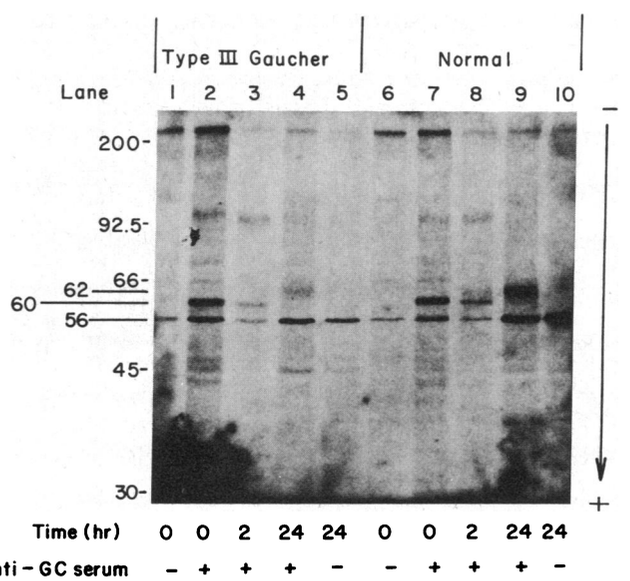

FIG. 3. $NaDodSO_4$ electrophoresis of immunoprecipitated fibroblast extracts from type III Gaucher disease (lanes 1–5) and normal (lanes 6–10). See Fig. 1 legend for details.

(6–8). The mature enzyme is also glycosylated, however, and we do not know whether sugars are added before or after cleavage of the leader sequence.

Thus, the initially labeled 60-kDa polypeptide could represent either the unglycosylated primary polypeptide or a partially glycosylated polypeptide from which the 19 or 39 amino acid leader had already been cleaved. This form of the enzyme then is gradually replaced by a still larger form, seen on the fluorograms as a broad band at 63 kDa. This is the mature enzyme; its position coincides with that of the purified, enzymatically active placental enzyme and with the main band of glucocerebrosidase antigen in cultured fibroblasts (4). The processing of the enzyme in the fibroblasts of six unrelated patients with type I Gaucher disease and one patient with type III Gaucher disease was precisely the same as normal: first a 60-kDa band and then the 63-kDa band appeared. Glucocerebrosidase processing in fibroblasts from patients with type II disease was quite different. The enzyme was obviously very unstable. None of the mature 63-kDa form could be found in any of the three cases examined.

The results of our studies of processing of glucocerebrosidase by fibroblasts of normal subjects and of patients with Gaucher disease differ significantly from those described by others. The results that have been reported earlier are also inconsistent with each other. Ginns *et al.* (3) originally suggested that normally a 63-kDa form of the enzyme was processed to a 56-kDa form and that this processing occurred more slowly than normal in type I Gaucher disease. Yet, in the same study they reported that in type I Gaucher disease the major form of enzyme found was at 56 kDa, precisely the opposite of what would be expected if processing to the lower molecular mass form was impaired. Subsequently, however, Jonsson *et al.* (9), performing pulse–chase experiments, reported that the earliest form of the enzyme had a molecular mass of 62.5 kDa, subsequently increasing to 66 kDa and then decreasing to a size of 59 kDa. In agreement with our present findings, no difference was found in that study between normal fibroblasts and those obtained from patients with type I Gaucher disease. At the same time, Erickson *et al.* (10) reported that the size of the earliest-labeled polypeptide was 60 kDa, converted subsequently to a 59-kDa fragment, then to a 56-kDa polypeptide, and finally to one with a molecular mass of 55 kDa. Most recently, however, Erickson *et al.* (11) reported that the progression in normal fibroblasts was from an initially labeled 60-kDa fragment, to 64 kDa (interpreted as a secretory form), and finally to 56 kDa and 55 kDa. The results of the various processing schemes reported by Ginns and his collaborators and those that we now find are summarized in Table 1.

The processing of normal fibroblast enzyme that we now observe is quite similar to that most recently described by Erickson *et al.* (11). However, we were unable to detect the 55-kDa degradation product that these authors described even in a chase carried out to 72 hr (data not shown). We readily detected a 56-kDa band in all of our gels, but its intensity was always similar in the half of extract immunoprecipitated with affinity-purified antiglucocerebrosidase serum beads and in the other half of the extract immunoprecipitated with preimmune rabbit serum beads. Thus, we considered this band to be unrelated to glucocerebrosidase. However, we cannot exclude the possibility that a glucocerebrosidase antigen with this molecular mass does exist and that it is obscured in our gels by the considerable amount of labeled immunoglobulin-binding polypeptide at this location. If it does exist, however, its quantity must be quite modest. Moreover, since the active form of the enzyme is 63 kDa in size, it is difficult to understand why degradation of active enzyme would "pause" at the 55-kDa level. Several differences between the non-neuronopathic type I Gaucher disease and the neuronopathic types II and III have been described. These include differences in enzyme kinetics (12), in the amount of cross-reactive antigenic material (13), and differences in posttranslational enzyme processing (3, 9). In the present study we find that the fibroblasts of three unrelated patients with type II Gaucher disease produced a very unstable precursor. In contrast, processing of glucocerebrosidase from patients with type I and type III Gaucher disease was normal. The latter finding is in disagreement with some earlier reports (3) but not with another (9).

We believe that our studies, using only affinity-purified antiserum and taking into account nonspecific binding of human fibroblast proteins, represent an accurate and internally consistent account of the processing of this enzyme.

Table 1. Processing of glucocerebrosidase by fibroblasts of normal subjects

Ref. or source	Enzyme, kDa			
	Form 1	Form 2	Form 3	Form 4
Ginns *et al.* (3)	63	56		
Jonsson *et al.* (9)	62.5	66	59	
Erickson *et al.* (10)	60	59	56	55
Erickson *et al.* (11)	60	64	56	55
This study	60	63		

This is publication no. 4457BCR from the Research Institute of Scripps Clinic. This work was supported in part by Grant 1-955 from the March of Dimes and Grant RR-00833 from the National Institutes of Health.

1. Saito, M., Mueller, O. T. & Rosenberg, A. (1984) *Gaucher Disease: A Century of Delineation and Research*, eds. Desnick, R. J., Gatt, S. & Grabowski, G. A. (Liss, New York), pp. 385–403.
2. Gravel, R. A. & Leung, A. (1983) *Hum. Genet.* **65**, 112–116.
3. Ginns, E. I., Brady, R. O., Pirruccello, S., Moore, C., Sorrell, S., Furbish, F. S., Murray, G. J., Tager, J. & Barranger, J. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5607–5610.
4. Beutler, E., Kuhl, W. & Sorge, J. (1985) *Am. J. Hum. Genet.* **37**, 1062–1070.
5. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
6. Sorge, J., Gelbart, T., West, C., Westwood, B. & Beutler, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5442–5445.
7. Sorge, J., West, C., Westwood, B. & Beutler, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7289–7293.
8. Ginns, E. I., Choudary, P. V., Tsuji, S., Martin, B., Stubblefield, B., Sawyer, J., Hozier, J. & Barranger J. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7101–7105.
9. Jonsson, L. V., Murray, G. J., Ginns, E. I., Strijland, A., Schram, A. W., Tager, J. M. & Barranger J. A. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 709 (abstr.).
10. Erickson, A. H., Ginns, E. I. & Barranger, J. A. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 709 (abstr.).
11. Erickson, A. H., Ginns, E. I. & Barranger, J. A. (1985) *J. Biol. Chem.* **260**, 14319–14324.
12. Grabowski, G. A., Dinur, T., Ostecki, K. M., Kruse, J. R., Legler, G. & Gatt, S. (1985) *Am. J. Hum. Genet.* **37**, 499–510.
13. Beutler, E., Kuhl, W. & Sorge, J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6506–6510.