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GALACTOSIALIDOSIS: A DIRECT EVIDENCE THAT A 46-KILODALTON PROTEIN RESTORES DEFICIENT ENZYME ACTIVITIES IN FIBROBLASTS

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The intracellular function of a specific protein to protect lysosomal β-galactosidase and neuraminidase activities against proteases in human fibroblasts was studied. β-Galactosidase was purified from human placenta to different degrees; a preparation (A) contained also two concomitant proteins, and a highly purified preparation (B) contained only the mature β-galactosidase. The protein concentrate of the culture medium of normal fibroblasts restored the activities of the deficient enzymes, β-galactosidase and neuraminidase, in galactosialidosis cells. This effect was inhibited only by the anti-A antiserum, and not by the anti-B antiserum. A 46-kilodalton protein, secreted from fibroblasts cultured in the presence of ammonium chloride, was detected again only by the anti-A antiserum, and not by the anti-B antiserum. It was concluded that this protein has a function to restore their activities in fibroblasts from galactosialidosis patients after being endocytosed from the culture medium.

Galactosialidosis is a human genetic lysosomal disease characterized by deficiency of β-galactosidase and neuraminidase activities in man (1). β-Galactosidase was partially restored in fibroblasts by protease inhibitors, and both enzyme activities were almost completely restored by the protein concentrate of the culture medium of normal or G_{M1}-gangliosidosis fibroblasts (2-5). D'Azzo et al (6) reported a defect of a 32-kilodalton (kDa) protein in fibroblasts from two patients with galactosialidosis. They detected this protein by immunoprecipitation using polyclonal anti-β-galactosidase serum.

However, there was no direct evidence that this 32-kDa protein, called as "corrective factor" (6) or "protective protein" (4), had actually the "protec-

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tive" effect on the deficient enzymes in this disease. In this study we used the antibodies against two different β-galactosidase preparations for differential evaluation of the enzyme protein and a low molecular protein in the culture medium of human fibroblasts.

Materials and Methods

Skin fibroblasts: The diagnosis of a 32-year-old male patient with galactosialidosis was established by typical clinical manifestations and biochemical data as reported previously (1). Skin fibroblasts from the patient and control subjects were cultured in Ham's F-10 medium supplemented with 10% fetal calf serum and antibiotics in a dish of 6 cm in diameter.

Purification of β-galactosidase and preparation of antibodies: β-Galactosidase was purified from human placenta according to the method of Lo et al (7), with minor modifications (preparation A). The final purification was performed by Octyl-Sepharose CL-4B(Pharmacia, Uppsala) chromatography (8), which resulted in a highly purified enzyme (preparation B). These enzyme preparations were used separately for raising antibodies; they were mixed with Freund's adjuvant (complete) and injected into rabbits. Consequently two different anti-β-galactosidase antisera (anti-A and anti-B) were obtained from these rabbits.

Preparation and assay of the culture medium concentrate: The protein concentrate containing "corrective factor" was prepared from the culture medium by the procedure reported by Hoogeveen et al (3). Confluent normal fibroblasts were cultured in 4 ml of F-10 medium containing 10 mM ammonium chloride for 2 days. The medium was dialyzed against phosphate-buffered saline and concentrated finally to 50 µl with a PM-10 Amicon membrane filter. Also the radiolabeled concentrate was prepared in the same way from the culture medium in the presence of [^{3}H]-leucine (Amersham, Buckinghamshire, England; 70 Ci/mmol, 0.2 µCi/dish of 10 cm in diameter) and ammonium chloride. Various dosages of the concentrate were added to the culture medium in a final volume of 4 ml in each dish, and intracellular enzyme activities were measured after cells were cultured for 3 days for the assay of the "corrective" effects.

Titration of anti-β-galactosidase antiserum: Culture medium concentrates were mixed with various dosages of the antibody in a volume of 300 µl and left overnight. Subsequently 10% protein A crude cell suspension (Sigma, St. Louis; 100 µl) was added. After incubation at 4°C for 60 min with shaking, the mixture was centrifuged at 10,000 x g for 5 min. The supernatant was added to the culture medium of galactosialidosis fibroblasts, and intracellular enzyme activities were measured after 3 days' cultivation.

Enzyme assays: β-Galactosidase and neuraminidase were assayed with fluorogenic substrates as described previously (2).

Immunoprecipitation: Immunoprecipitation of the radiolabeled protein concentrate was performed according to the method of Hasilik and Neufeld (9), using two anti-β-galactosidase antisera described above. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 10% slab gels (10), and radioactive bands were visualized by fluorography.

Results

The preparation A after the initial purification procedures of β-galactosidase contained three protein bands (64-, 28-, and 20-kDa) (Fig. 1A). Only a 64-kDa protein was detected after the final preparation by Octyl-Sepharose

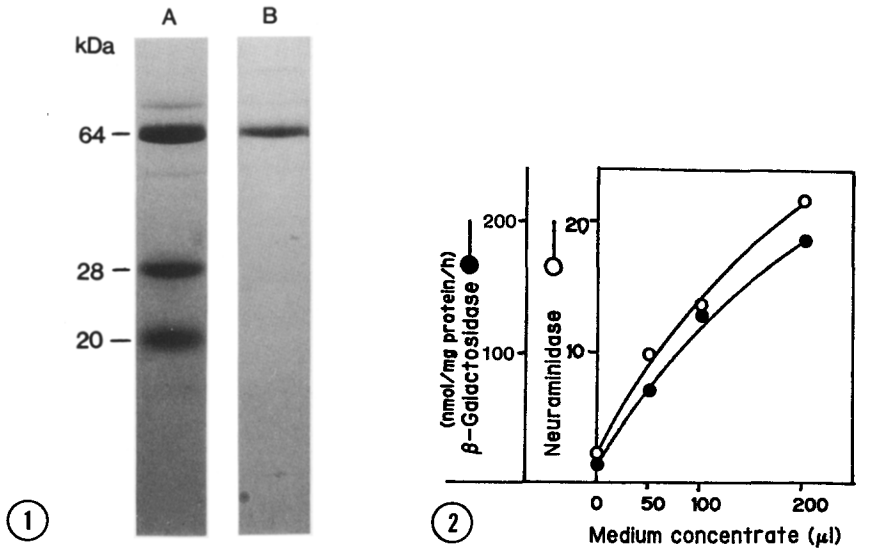


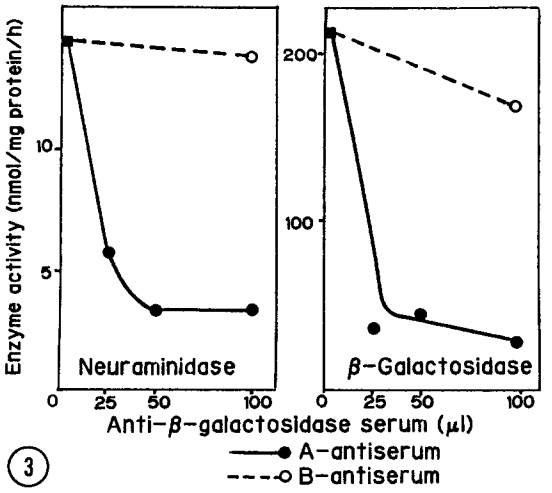
Fig. 1. Polyacrylamide gel electrolphoresis in the presence of sodium dodecyl sulfate of two β-galactosidase preparations in different degrees of purification. Preparation B was purified by Octyl-Sepharose CL-4B chromatography from preparation A.

Fig. 2. Restoration of the deficient enzymes in galactosialidosis fibroblasts by adding the medium protein concentrate. See Materials and Methods for experimental details.

CL-4B chromatography (preparation B) (Fig. 1B). Anti-A and anti-B antisera were used for further differential analysis.

In galactosialidosis cells both β-galactosidase and neuraminidase activities were markedly restored after incubation for 3 days in the culture medium containing the medium protein concentrate. This effect was increased in proportion to the amount of the concentrate added (Fig. 2), and was used for assays of the "corrective" activities. Immunotiters of the anti-A and anti-B antisera were monitored by this assay system on the medium protein concentrate. The effects of these anti-β-galactosidase antisera are shown in Fig. 3. The restoration of β-galactosidase and neuraminidase activities in galactosialidosis cells was almost completely inhibited by the anti-A antiserum. The anti-B antiserum had little or no effect on these enzyme activities.

Immunoprecipitation of the radiolabeled medium protein concentrate (Fig. 4) revealed two different patterns with these antisera against the specific β-galactosidase preparations. The 92-kDa precursor of β-galactosidase was de-



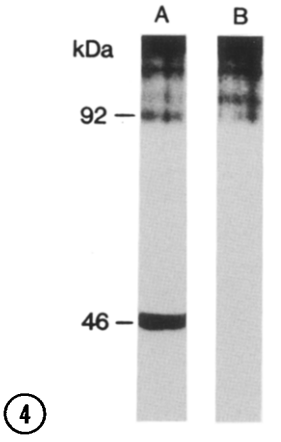


Fig. 3. Immunotitration of the medium protein concentrate using two different anti-β-galactosidase sera. See Materials and Methods for experimental details.

Fig. 4. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of radiolabeled medium protein concentrate of normal fibroblasts, immunoprecipitated with anti-A antiserum (lane A) and with anti-B antiserum (lane B).

tected by both antisera. However, the 46-kDa protein, demonstrated by the anti-A antiserum, was not precipitated and visualized by the anti-B antiserum.

Discussion

D'Azzo et al (6) reported the absence of a specific 32-kDa protein as the basic molecular abnormality of galactosialidosis. However, this finding has not been confirmed in any other reports although we observed the same abnormality in one case by the use of the anti-β-galactosidase antibody which had been prepared by investigators in Rotterdam (1). However, the antibodies prepared previously in our laboratory did not reveal the same abnormality in any of the cases of this disease. Futhermore, the direct connection has not been shown between this 32-kDa protein revealed by immunoprecipitation and the "corrective" effect on the enzyme activities in the previous reports.

The purified β-galactosidase from human placenta yielded two homogeneous isozymes (7), and the purified β-galactosidase used for immunization in the report of D'Azzo et al (6) seemed to be a mixture which contained a smaller protein in molecular size. In our experiment, we obtained two β-galactosidase

preparations in different degrees of purification. Antibodies were raised against each of them and used for further analysis.

Only the preparation containing small molecular proteins (preparation A) reacted with the 46-kDa protein, together with the precursor molecule of β-galactosidase, in the culture medium. It also specifically inhibited the effect to restore enzyme activities in galactosialidosis cells by the concentrate of the culture medium. These results indicated that only this anti-A antiserum contained the anti-"corrective factor" antibody as well as the anti-β-galactosidase antibody. From these data it is concluded that this 46-kDa protein is endocytosed from the culture medium by galactosialidosis fibroblasts, processed into the 28- and/or 20-kDa mature protein(s) (Nanba et al, unpublished data), and works as a stabilizer and activator of the deficient enzymes, β-galactosidase and neuraminidase, in the cells. Further studies are necessary to identify the protein molecule which actually has the "protective" and "corrective" activities toward these two enzymes intracellularly.

Acknowledgments

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