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New Method for the Cytochemical Demonstration of Aspartylglucosaminidase Activity

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A new method for the cytochemical demonstration of aspartylglucosaminidase (AGA, EC 3.5.1.26) is proposed. The method employs a newly designed substrate-L-aspartic acid-4-(1-hydroxy-4-naphthylamide).HCl (Asp-HNA),which, upon the enzyme hydrolysis, releases a highly active reducing agent - 4-amino-1-naphthol. The last compound reduces immediately and quantitatively the tetrazolium salt Nitro Blue Tetrazolium (NBT) in the incubation medium to an water-insoluble, deeply colored formazan, precipitating at the sites of the enzyme activity and marking them accurately. The method is successfully applied to the visualization of AGA in cultured human lung fibroblasts.

Key words: Aspartylglucosaminidase, Tetrazolium method, Enzyme cytochemistry.

Introduction

Aspartylglucosaminidase (AGA, EC 3.5.1.26) is a lysosomal amidohydrolase, involved in the intracellular degradation of N-glycoproteins. It hydrolyzes the amide bond between the β-carboxyl group of L-aspartic acid and the 1-amino group of 1-amino-2-acetamido-1,2-dideoxy-β-D-glucose at the reducing end of the oligosaccharide chain in N-glycoproteins. Latterly, the unstable 1-aminoglycan decomposes non-enzymatically to ammonia and oligosaccharide, possessing N-acetyl-glucosamine as a terminal moiety [9]. The enzyme recognizes the free α-amino and α-carboxyl groups of L-aspartic acid, i.e. it acts only on glycoasparagines, obtained after the completion of the proteolytic digestion [8]. AGA is not specific towards the carbohydrate chain, which might be attached to the N-acetylglucosamine moiety [12]. In contrast to the majority of the lysosomal hydrolases, AGA is found to have an alkaline pH-optimum of 7.6 [7, 9]. AGA hydrolyzes a series of compounds such as L-Asp-β-methyl ester, L-Asp-β-hydroxamate, etc. [8] as well as artificial substrates, e.g. L-Asp-β-(7-amido-4-methylcoumarin) [11]. AGA activity is reported to be high in the human fibroblasts [5]. In humans, the genetically determined deficiency of AGA results in a lysosomal storage disease aspartylglycosaminuria (AGU). The only clustering of AGU-patients

is limited in Finland, whereas in other European countries AGU-cases are rare (10).Using polyclonal antibodies AGA has been demonstrated in the lysosomes of human fibroblasts in healthy people, the same technique showing a lack of the enzyme in the lysosomes of AGU-patients fibroblasts [5]. No method for the cytochemical detection of AGA has been proposed so far.

In the present study we propose a method for the cytochemical demonstration of AGA, based on a newly synthesized substrate — L-aspartic acid-4-(1-hydroxy-4-naphthylamide).HCl (Asp-HNA), employed according to the tetrazolium principle. We also describe the visualization of the enzyme in cultured human lung fibroblasts by the new method.

Material and Methods

Synthesis of the substrate. The 4-amino-1-naphthol hydrochloride was synthesized as described previously [3, 4]. It was bound to N-benzyloxycarbonyl-aspartic acid-α-benzyl ester (Z-Asp-OBzl) (Bachem, Switzerland) according to the dicyclohexylcarbodiimide method [1] using N-ethylmorpholine as HCl-acceptor in an argon atmosphere. The amino acid protective groups — the Z-group and the benzyl ester were cleaved simultaneously by a catalytic hydrogenation in ethanol/HCl with palladium on activated carbon as a catalyst. Thus, the AGA substrate was obtained as a HCl-salt-L-aspartic acid-4-(1-hydroxy-4-naphthyl-amide).HCl (Asp-HNA).

Cell cultures and incubation medium. Human lung fibroblasts, cell lines F 898 and P 742 were grown in diploid medium (Sigma, USA), containing 10 % normal bovine serum on cover slides at 37 °C for 72 hours. After the cultivation, the cells were washed in PBS, air-dried and incubated in a substrate medium, consisting of 5mM substrate (Asp-HNA) and 1mg/ml NBT (both the substrate and tetrazolium salt were pre-dissolved in minimal volumes of dimethylformamide), diluted with 0.1 M phosphate buffer, pH 7.6, at 37 °C for 45 to 60 minutes. After the incubation the cells were post-fixed in 4 % neutral formaldehyde and embedded in glycerol-jelly.

Cells, treated in the same way, but incubated in a substrate-free medium (phosphate buffer, containing NBT) were used as controls.

Results and Discussion

No precipitates of the final reaction product (the NBT diformazan) were observed in the controls. In the cultured cells, the final reaction product was deposited in the form of fine granules, which coincided with the lysosomal localization of the enzyme. The AGA activity proved to be high in human lung fibroblasts, cell lines P 742 and F 898, which was demonstrated by the numerable bluish-violet formazan granules occupying the whole cell cytoplasm (Fig. 1 and 2).

Presently, the lysosomal enzyme aspartylglucosaminidase is a subject of an increasing interest. The enzyme plays a crucial role in the last stages of glycoprotein degradation in mammalian cells. The congenital deficiency of AGA in humans results in AGU disorder, which is characterized by a severe mental retardation and typical facial and skeletal abnormalities [10]. Thus, a convenient method for the cytochemical demonstration of AGA would be much useful for the study of the enzyme properties. Unfortunately, by now the enzyme has been assayed only biochemically using L-Asp-β-(7-amido-4-methylcoumarin) as a fluorogenic substrate (11). However,

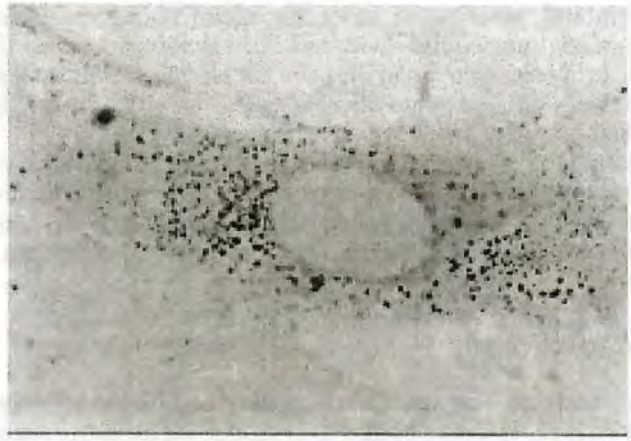

Fig. 1. Cell culture of human lung fibroblasts, line P 742. Deposits of numerous fine formazan granules, consistent with the lysosomal localization of the enzyme (× 600)

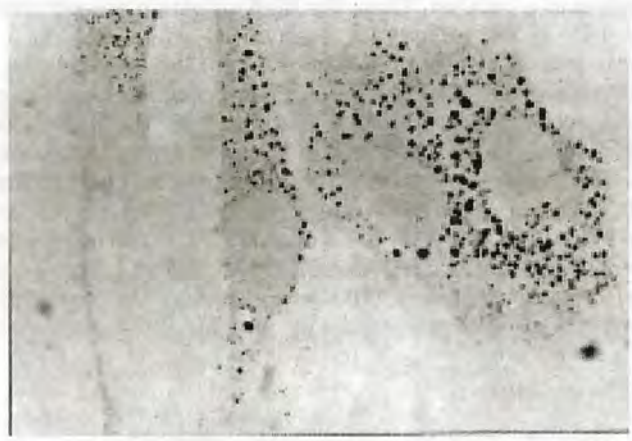

Fig. 2. Cell culture of human lung fibroblasts, line F 898. High activity of AGA, visualized by the numerous formazan granules within the cells cytoplasm (× 600)

the same substrate could not be applied for the cytochemical visualization of the enzyme, because the fluorescent leaving group 7-amino-4-methylcoumarin tends to be highly water-soluble. Furthermore, the immunocytochemical methods are very expensive, requiring to create highly specific antibodies against the enzyme molecules [5].

The AGA substrate, which we synthesized, proved to be very convenient for the cytochemical localization of the enzyme. It is readily hydrolyzed by AGA; the liberated 4-amino-1-naphthol reduces rapidly and quantitatively the tetrazolium salt to formazan, which precipitates on the enzyme activity locations. Our results for the lysosomal localization of AGA are consistent with the previous findings, that the enzyme activity is restricted to the lysosomal fraction at differential centrifugation of

tissue homogenates and that it is to be solubilyzed by detergents (2, 6). The immunocytochemical methods, mentioned above,show also that the enzyme is localized in the lysosomes. Using the newly proposed method for AGA we demonstrated the enzyme in the lysosomes of human cells. The method, proposed by us could be exploited in the clinical diagnostic of people with congenital deficiency of the enzyme.

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