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Attempts to localize histochemically aspartylglucosaminidase

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A direct tetrazolium salts method for the histochemical localization of Aspartylglucosaminidase (AGA) is proposed. The method is based on the substrate *L*-aspartic acid-β-menadiol ester. The precise localization of the enzyme in different rat organs can be revealed by the new method. For the first time AGA is localized histochemically in tissue sections.

Key words: Aspartylglucosaminidase, Menadiol, Tetrazolium salts method, Enzyme histochemistry.

Introduction

N-glycoproteins represent complex biochemical compounds, in which the oligosaccharide chains are bound to the protein by an amide bond between 1-amino-N-acetylglucosamine and the β-carboxyl group of *L*-aspartic acid. In animal cells this amide bond is hydrolysed by N-aspartyl-β-N-acetylglucosamineamidohydrolase (aspartylglucosaminidase, AGA, EC 3.5.1.26), a specific enzyme of the amidohydrolase group. The enzyme acts only on substrates with a single aspartic acid residue, i. e., after the proteolytic digestion of the protein chain. AGA has been isolated and purified from different human and animal organs, e. g. from rat [1,2] and human [3, 4, 5] liver and its biochemical properties have been studied extensively. The genetically determined deficiency of the enzyme, i. e. the aspartylglucosaminuria disease results in the secretion of uncleaved aspartylglucosamines into the urine. Using polyclonal antibodies AGA has been localized in the lysosomes of the fibroblasts in healthy humans, but has not been found in patients, suffering from aspartylglucosaminuria [6]. Recent investigations on AGA show, that the enzyme can act not only as an amidohydrolase but as an esterase as well. For example, AGA hydrolyses the β-methylester of Asp [7]. This property of the enzyme has prompted us to synthesize the β-esters of Asp with 1- and 2-naphthols and menadiol and to use these compounds as substrates for the histochemical localization of AGA. For the same purpose we have synthesized the β-amide of Asp with 1-naphtylamine.

Materials and Methods

1. *Synthesis of the substrates.* The synthesis of the substrates was carried out using Z-Asp-α-benzylester and 1-naphthol, 2-naphthol, menadiol or 1-naphthylamine in tetrahydrofuran with dicyclohexylcarbodiimide. After the purification of the substances obtained during the reaction, the protective groups of Asp were cleaved with H_2/Pt on activated carbon.

The specific inhibitor of AGA, 5-diazo-4-oxo-L-norvaline (DONV), was synthesized according to H a n d s c h u m a c h e r et al. [8].

2. *Animals.* Wistar rats of both sexes were used. After decapitation in deep ether anaesthesia, samples from different organs were fixed in 2 % formaldehyde (freshly prepared from paraformaldehyde) in 0,1 M phosphate buffer, pH 7,6 for 24 hours at 4 °C. Then, the pieces were washed for 24 hours in Holt's solution, frozen in liquid nitrogen and 10 μm cryostat sectons were cut.

3. *Incubation solutions.* The cryostat sections were incubated for 1 hour at 37 °C in the following incubation solutions: 1 to 3 mM *L*-aspartic acid-4-(1-naphthyl)-ester (Asp-1-NE) or *L*-aspartic acid-4-(2-naphthyl)-ester (Asp-2-NE) and 1 mg Fast Blue B/ml in 0,1 M phosphate buffer, pH 7,2; 1 to 3 mM *L*-aspartic acid-4-(1-naphthyl)-amide (Asp-NA) and 0,03 ml freshly diazotized New Fuchsine/ml in 0,1 M phosphate buffer, pH 6,5; 1 to 3 mM *L*-aspartic acid-4-monomenadiolester (Asp-ME) and nitro blue tetrazolium (NBT) or tetranitro blue tetrazolium (TNBT) in 0,1 M Tris buffer, pH 7,6. After incubation the sections were post-fixed in 4 % neutral formaldehyde, washed with water and embedded in glycerol-gelatine. Parallel sections were incubated in the same medium in the presence of 200 μM DONV. Additonally, other sections were incubated without the AGA substrates and termed as controls or with menadiol diacetate and NBT for the demonstration of non-specific esterases after D i k o w and G o s s r a u [9] with or without the AGA inhibitor DONV.

Results

The best results were obtained with the substrate Asp-ME, employing the tetrazolium salts method. The reaction products, i. e., the diformazans of NBT or TNBT, were localized as fine granules in the epididymal principle and clear cells as well as in spermatozoa (Fig. 1), hepatocytes (Fig. 2), epithelial cells of the proximal renal tubules (Fig. 3), macrophages and reticular cells of the spleen, enterocytes of the intestine (Fin. 4) and in giant lysosomes of the ileum of 7-day-old rats (Fig. 5). In the presence of the AGA inhibitor DONV the enzyme activity was almost completely suppressed in the epididymis, liver, kidney and spleen but in the intestine of suckling and adult rats AGA activity was unchanged.

Using Asp-1-NE or Asp-2-NE as substrates, the azo-dye showed the same localization of AGA as with Asp-ME, however the contrast was considerably lower. No reaction product was observed when Asp-NA was used as a substrate. The demonstration of non-specific esterases with menadiol diacetate and NBT showed a similar localization in the studied organs as with the AGA substrates, but the enzyme activity did not change in the presence of DONV.

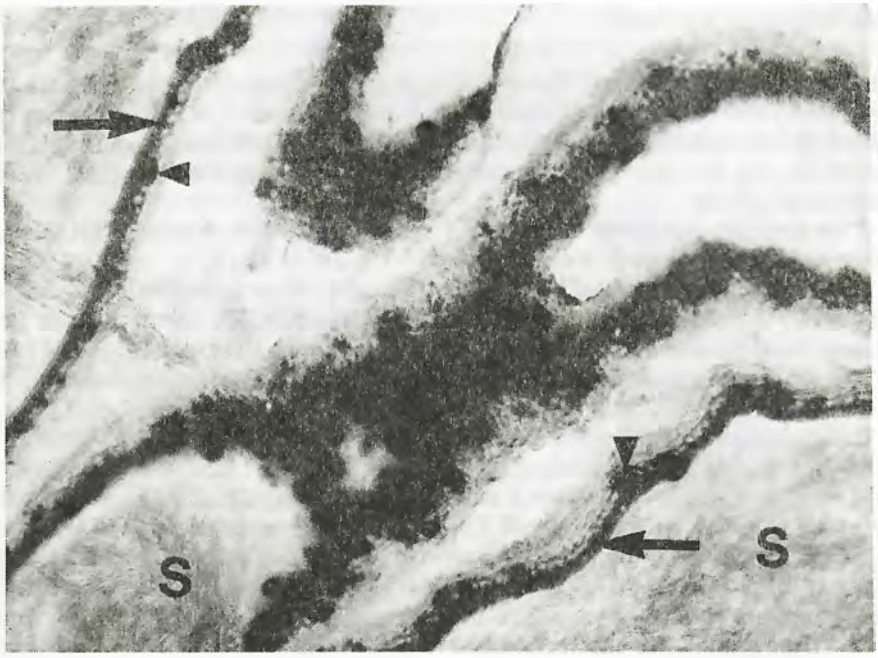

Fig. 1. Rat epididymis. Demonstration of AGA with Asp-ME as a substrate and NBT. The granulated reaction product — NBT diformazan appears in the principle (arrows) and clear (arrowheads) cells and in spermatozoa (S). × 400

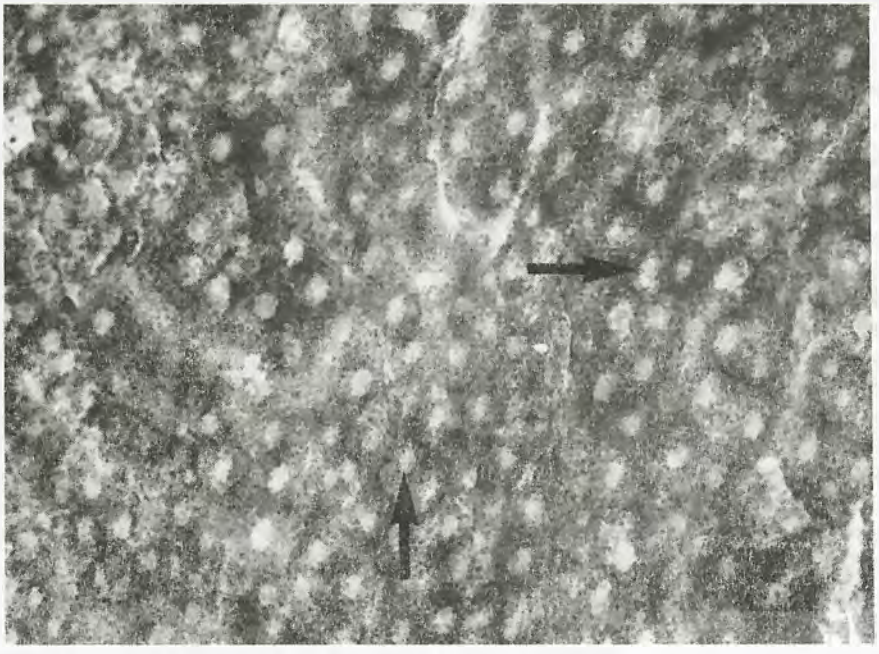

Fig. 2. Rat liver. Demonstration of AGA activity with Asp-ME and NBT. The reaction product — NBT diformazan is present in hepatocytes (arrows). × 400

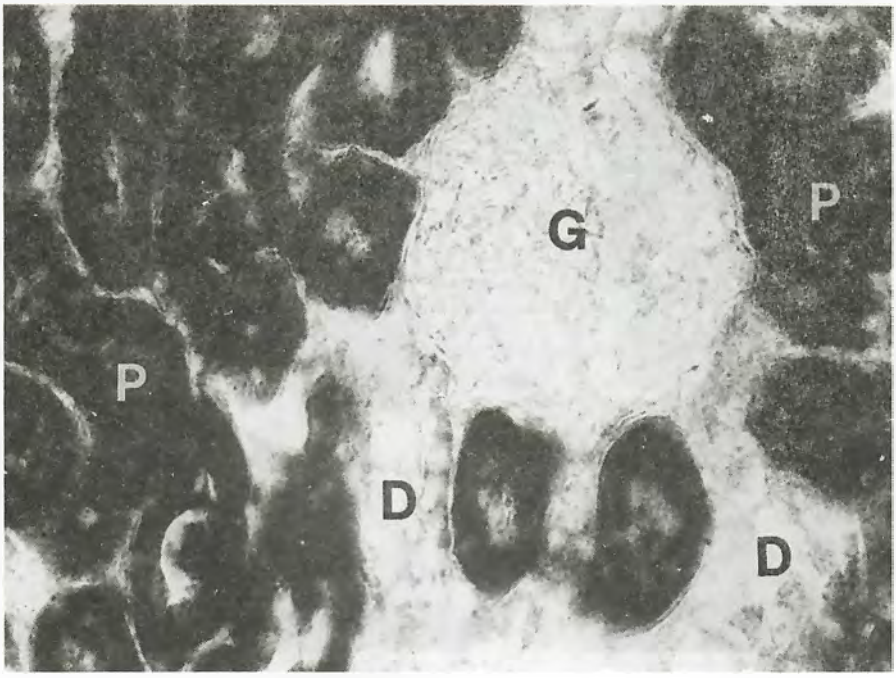

Fig. 3. Rat kidney. Demonstration of AGA with Asp-ME and TNBT. The reaction product – TNBT diformazan is visible in the epithelial cells of the proximal renal tubules (P). ×400
G – glomerulus; *D* – distal tubules

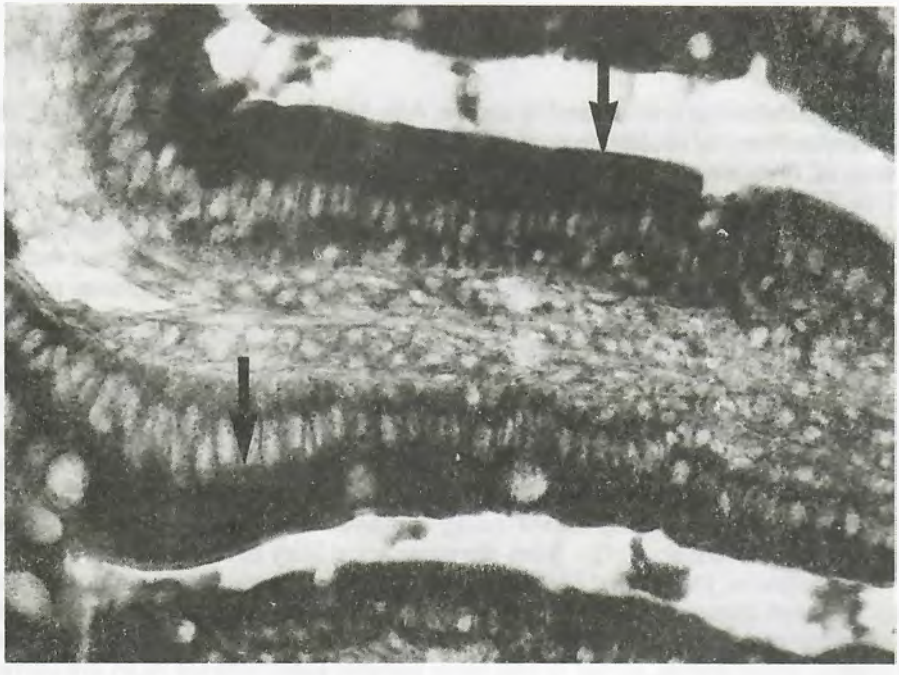

Fig. 4. Rat intestine. AGA demonstration with Asp-ME and NBT. High amounts of NBT diformazan are seen in the enterocytes (arrows). ×400

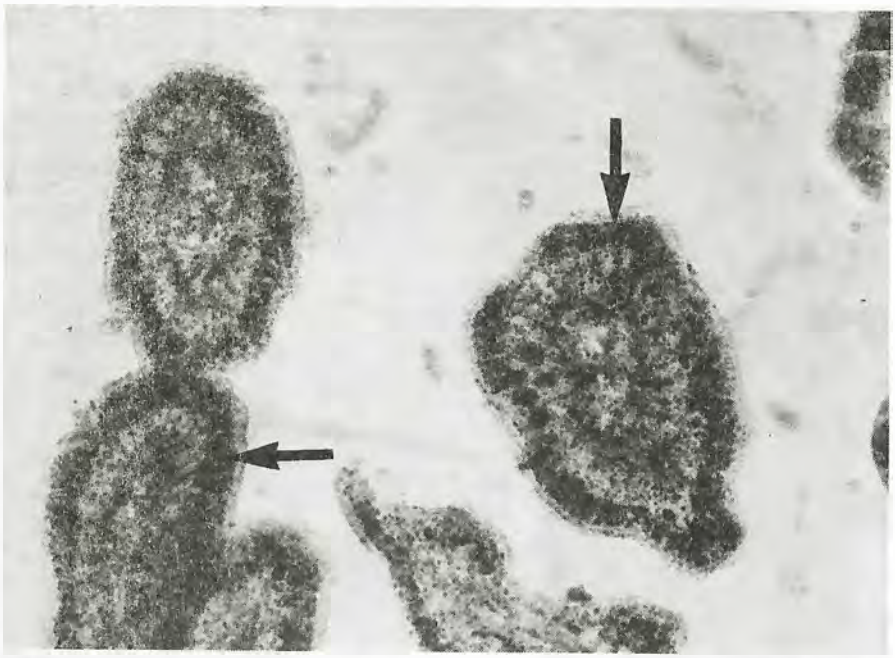

Fig. 5. Ileum of a 7-day-old rat. Visualization of AGA activity with Asp-ME and TNBT. TNBT diformazan is present in the region of giant lysosomes (arrows) of the enterocytes. ×400

Discussion

One of the substances synthesized by us, i. e. Asp-ME appeared to be a good substrate for the histochemical localization of AGA. This compound was readily hydrolysed at the sites of lysosomes in many cells of rat tissues; the liberated menadiol reduced NBT or TNBT to intensively coloured diformazans, thus marking the sites of the enzyme activity. The staining was almost completely inhibited by the specific AGA inhibitor DONV in most of the studied organs, but no inhibition was observed in the rat intestine. The histochemical demonstration of non-specific esterases using the same tetrazolium salts procedure, but with a different substrate (menadiol diacetate) showed co-localization with AGA and non-specific esterase activity was not suppressed by the AGA inhibitor DONV. These data sugest, that the enzyme activity, remaining after AGA inhibition by DONV could be ascribed to non-specific esterases, which partially also hydrolysed Asp-ME. Therefore, when Asp-ME is used as a substrate for the histochemical localization of AGA, the co-reaction of non-specific esterases has always to be considered and gives the method only a limited specificity. On the other hand, all biochemical assays using synthetic substrates, e. g. aspartic acid amidomethylcoumarines suffer from the same specificity problems.

In conclusion, for the first time an enzyme of the N-glycoproteinamidohydrolases group has been demonstrated histochemically, which provides us with the possibility to obtain more information about AGA in heterogenously structured cells, tissues and organs. Experiments, aimed to find more suitable substrates for AGA are currently in progress in our laboratories.

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