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Fluorescent Histochemical Localization of Dipeptidyl Aminopeptidase IV Using a Newly Developed Substrate

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A new fluorogenic substrate for histochemical localization of dipeptidyl aminopeptidase IV / CD26 (DPP IV; EC 3.4.14.5) is synthesized — Gly-L-Pro-6-hydrazido-2-hexyl-1,8-naphthalimide and applied for the histochemical visualization of the enzyme in tissue sections of rat organs. Upon the enzyme action the substrate is hydrolyzed and the fluorochrome N-hexyl-6-hydrazino-1,8-naphthalimide is liberated, which reacts instantly with an aromatic aldehyde, e.g. piperonal (3,4-methylenedioxybenzaldehyde; PPL), presented in the incubation medium to form a water-insoluble orange hydrazone, which fluoresces in brilliant red when excited by green light (λexcit = 540 nm; λemiss = 620 nm). The enzyme locations are visualized by red fluorescence at a minimal or none background noise. The newly developed histochemical technique is used successfully for the fluorescent detection of DPP IV in all its locations, including the capillary bed endothelium, which has not been achieved by now.

Key words: Dipeptidyl aminopeptidase IV, naphthalimide derivatives, fluorescent histochemical methods, synthetic enzyme substrates, enzyme histochemistry.

Introduction

Dipeptidyl aminopeptidase IV (DPP IV, EC 3.4.14.5) is a serine type membrane-associated exopeptidase with ubiquitous expression. The enzyme hydrolyzes preferably X-Pro dipeptides from the amino-terminal of different oligo- and polypeptides at pH optimum 7.8 [5]. The enzyme is recognized as cell differentiation factor CD 26, expressed in T lymphocytes plasmalema [8] DPP IV plays an important role in regulatory processes such as T-cell activation, cell migration and invasiveness [10], digestion of proline containing peptides (mainly collagen) [1], etc. According to some present studies the enzyme was proposed as a specific marker for malignant transformations in the kidney [14]. Aberrant DPP IV expression was also detected in human hepatocellular carcinoma [15]. Obviously, the enzyme has a very important role in different biological processes and reasonably a lot of research groups are interested of it.

The histochemical visualization of DPP IV in normal and pathologically altered tissues is now performed exclusively by chromogenic methods [6]. There are also fluoro-

genic substrates, e.g. X—Pro-rhodamide 110 [11], Ala-Pro-2-cresyl violet [16] and Gly-Pro-4-methoxy-2-naphthylamide [7] but they are applicable for the enzyme detection only in cultured cells and not in tissue sections.

In the present paper we propose a new fluorogenic substrate for dipeptidyl aminopeptidase IV - Gly-L-Pro-6-hydrazydo-2-hexyl-1,8-naphthalimide, synthesized by us, and a novel fluorescent technique for the enzyme localization, based on it. Using the here presented histochemical method we visualized the enzyme in tissue sections of different rat organs.

Materials and Methods

Synthesis of the fluorochrome. The fluorochrome N-hexyl-6-hydrazino-1,8-naphthalimide (HHNI) was synthesized as follows: Acenaphthene (Merck, Germany) was brominated by N-bromosuccinimide in dimethylformamide after R o s s et al. [13]. The obtained 6-bromo acenaphthene was oxidized to 6-bromonaphthalanhydride by a standard procedure with sodium dichromate in acetic acid. The last compound was coupled with hexylamine by boiling it in absolute ethanol for 12 hours to give 6-bromo-N-hexyl-1,8-naphthalimide [2]. The fluorochrome — HHNI was obtained from the brom-containing compound and hydrazine monohydrate in dimethylsulphoxide at 60°C using potassium fluoride as auxiliary reagent and tetrabutylamonium sulfate as a catalyst by a novel procedure, which will be published elsewhere.

Synthesis of the substrate. The DPP IV substrate — Gly-L-Pro-6-hydrazido-2-hexyl-1,8-naphthalimide (Gly-L-Pro-HHNI) was synthesized from HHNI and Boc-Gly-L-Pro (Bachem, Switzerland) by N,N,N′,N′-Tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) procedure [9]. The Boc-protection was cleaved in 4N HCl/ dioxane to obtain the substrate as hydrogen chloride salt.

Tissue treatment and incubation. Mature Wistar rats of both sexes were decapitated under ether anesthesia. Pieces of kidney, small intestine, epididymis, liver, heart and spleen were removed and frozen immediately in liquid nitrogen. Ten μm thin sections were cut on cryotome Reichert Jung (Nussloch, Germany) at –25°C and mounted on gelatinized glass slides. Half of them were covered with 0.75 % celloidin for one minute at room temperature; the other sections were left free of celloidin film. Both types of sections were incubated in liquid solutions, consisting of 0.25 to 0.5 mM of the substrate (Gly-L-Pro-HHNI) and 1 mg/ml aromatic aldehyde (piperonal, anizaldehyde, terephthalic aldehyde or antracen-9-carbaldehyde), dissolved in buffer (0.1 M HEPES, 0,1 M phosphate or 0,05-borax/boric acid), at pH 7.8. The incubation lasted for 60-120 min at 37°C, because of the different enzyme activity levels in the examined rat organs. All the samples were post-fixed in 4 % neutral formaldehyde and embedded in glycerol-jelly. Control sections were incubated only in buffered aldehyde (in the absence of the substrate).

The sections were studied under fluorescent microscope OPTON IM 35 with filter combination G 546 FT 580 LP 590. The photos were made on Konica Minolta (Japan) VX 200 colorful films.

Results and Discussion

6-Substituted-1,8-naphthalimides are highly fluorescent compounds, which are used as dyes for cotton, wool, silk, synthetic fibers and plastics [12]. The 6-hydrazino-2-alkyl-1,8-naphthalimides are found to possess unique electrochemical properties and, though recently synthesized, are already used in conducting and magnetic materials as well as in

nanotechnology [17]. They fluoresce in green when excited by blue light (λexcit = 430 nm, λemiss = 540 nm) [3], whereas their hydrazones with aromatic aldehydes, possessing an electron donor group at para-position show a large batochrome shift in the fluorescent spectra (λexcit = 540-580 nm, λemiss > 600 nm), i.e. they fluoresce in red when excited by green light. In the present study, we use the unique properties of these compounds to develop a novel fluorescent histochemical procedure for the visualization of dipeptidyl aminopeptidase IV on the basis of the substrate Gly-L-Pro-HHNI, synthesized by us. Our investigations show that the enzyme hidrolyzes the substrate, and as a result the fluorochrome 6-hydrazino-2-hexyl-1,8-naphthalimide, which is the primary reaction product, is liberated. Fluorescence of the last compound coincides with the tissue autofluorescence. To avoid this, we use aromatic aldehyde, as a coupling agent, to obtain orange hydrazone, which marks the places of enzyme activity. From the tested aromatic aldehydes, best results were achieved with piperonal. Its hydrazone shows stable red fluorescence, when excited with green light (λexcit 540-580nm). At this wavelength, tissue fluorochromes are not excited and we observed enzyme localization at the lack of background noise. The best visualization results were obtained in 0,1 M phosphate buffer. Sixty minutes of incubation was needed in organs where the enzyme activity is high (liver, kidney, small intestine). DPP IV activity was observed respectively in the glomeruli and in the brush-borders of convoluted tubules in kidney (Fig. 1), strictly in the brush border of small intestinal enterocytes (Fig. 2), and there is a reaction product in bile canaliculi and sinusoids in the liver (Fig. 3). In epididymis and spleen the enzyme activity is lower and ninety minutes of incubation period was necessary. DPP IV activity was visualized in the epididymis the reaction was seen in principal and basal cells of the channels (Fig. 4) and in the spleen the

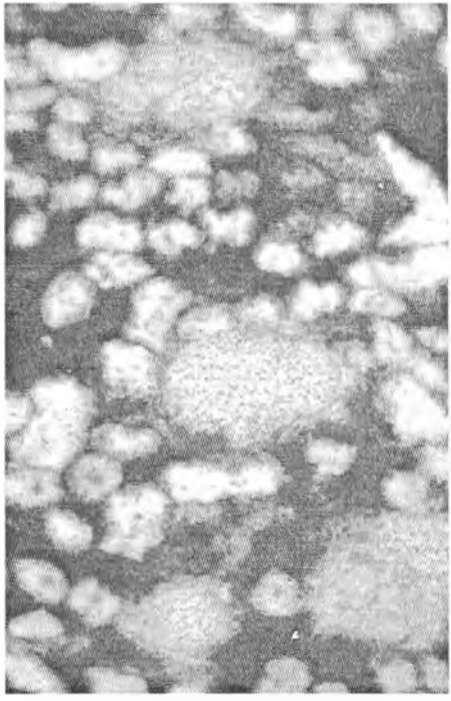

Fig. 1. Kidney. DPP IV activity in glomeruli and in the brush-border of convoluted tubules (× 200)

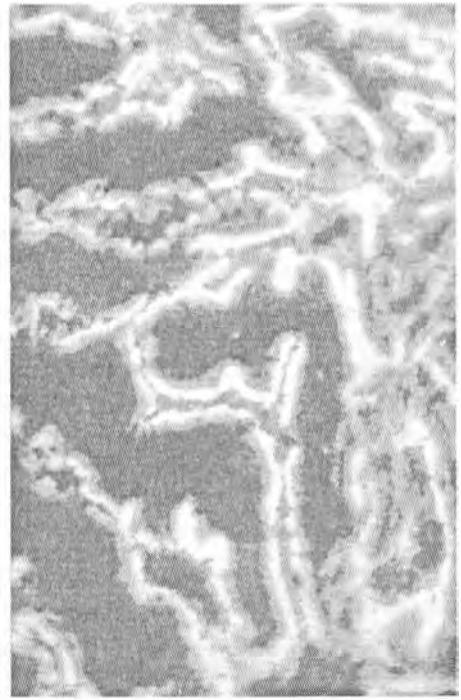

Fig. 2. Small intestine. The reaction product is strictly localized in the brush border of small intestinal enterocytes (× 200)

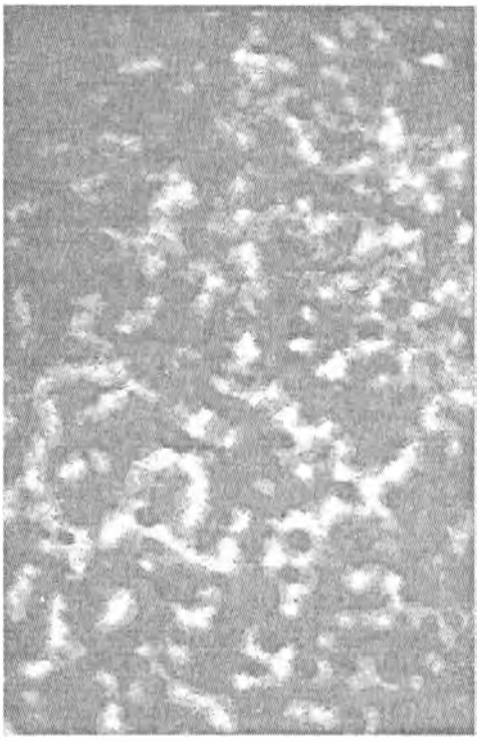

Fig. 3. Liver. Enzyme reaction in bile canaliculi and liver sinusoids (× 500)

Fig. 4. Epididymis. The reaction is seen in principal and basal cells of the channels (× 200)

Fig. 5. Spleen. Reaction product in the sinusoids of the red pulp and in the surrounding lymphocytes, no reaction in the white pulp (× 500)

reaction product was localized in sinusoids of the red pulp and in the surrounding lymphocytes; no reaction was detected in the white pulp (Fig. 5).

In a previous paper [4], we proposed synthetic fluorescent substrate for amino peptidase N — L-Ala-6-hydrazido-2-butyl-1,8-naphthalimide in a similar visualization procedure, by which we obtained a strict visualization of the enzyme in all its locations. In comparison with this technique, the present substrate possesses a longer alkyl chain and the final reaction product — the hydrazone, has even more amorphous appearance. An important achievement of the here proposed synthetic fluorescent substrate is that it permits to visualize DPP IV activity in the glomeruli, sinusoid capillaries of spleen red pulp, liver sinusoids and bile canaliculi and in heart capillaries, which has not been done so far with the known fluorescent substrates [6].

The novel histochemical procedure for DPP IV visualization, proposed by us can be used to detect enzyme activity in all its locations and to compare qualitatively the enzyme levels in normal and pathologically altered tissues and organs.

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