Immunohistochemical localization of 5-oxo-L-prolinase, an enzyme of the γ-glutamyl cycle, in porcine brain microvessels

Martina Jäger[a], Peter Weber[b], Sabine Wolf[c,*]

[a] *Max-Planck-Institut für Immunbiologie, Stübeweg 51, Freiburg 79108, Germany*
[b] *CRC Laboratories, Institute for Cancer Studies, Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TJ, UK*
[c] *Institut für Biochemie, TU-Darmstadt, Petersenstr. 22, Darmstadt 64287, Germany*

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Abstract The immunohistochemical analysis of the distribution of 5-oxo-L-prolinase in porcine brain at the light microscopic level was performed with an antibody raised against the enzyme purified from pig kidney. The present study reveals the specific expression of 5-oxo-L-prolinase in brain capillaries with an average diameter of 4.1 ± 0.9 μm, while larger blood vessels remain unstained. Porcine kidney and skeletal muscle show no endothelial-specific staining with the antibody. In some cases, the asymmetrical staining pattern in cross and longitudinal sections of brain microvessels indicate endothelial- but also pericyte-specific expression.

Key words: 5-Oxo-L-prolinase; γ-Glutamyl cycle; Blood-brain barrier; Brain microvessel endothelial cell; Pericyte; Amino acid transport

1. Introduction

In most vertebrates, homoeostasis of the brain is maintained by the blood-brain barrier (BBB), a regulatory interface localized in the continuous layer of brain microvessel endothelial cells (BMECs) [1,2].

A commonly used marker of the BBB is γ-glutamyl transpeptidase (GGT: EC 2.3.2.2), an enzyme not found in the endothelium of peripheral tissues. In organs such as liver and kidney, GGT plays a role in amino acid transport, metabolism of leukotrienes and conversion of toxic substances to mercapturic acids [3]. When GGT expression was shown in brain capillaries, it was initially proposed that the enzyme might participate in the delivery of amino acids into the central nervous system via the γ-glutamyl cycle [4]. In more recent studies, GGT was mainly found in abluminal membranes of BMECs and microcapillary associated pericytes [5,6] and was described to contribute to pericytes' ability to act as a second line of defense catabolizing brain-borne leukotriene C4 [7]. Interestingly, species-dependent differences in the localization of GGT at the blood-brain interface were observed [8]. The enzyme was present on the luminal surface of endothelial cells in rats, whereas in mouse, monkey and human cortex, GGT was found in astrocytic endfeet around the vessels. The authors concluded that in the latter case, GGT from astrocytes may play a role in the detoxication of lipophilic xenobiotics that cross the endothelial barrier [8].

The amino acid transport hypothesis was supported by Lee and coworkers [9], who demonstrated that 5-oxo-L-proline, an intermediate of the γ-glutamyl cycle and the substrate of 5-oxo-L-prolinase (5-OPase), acts as a second messenger molecule, stimulating the amino acid transport across the BBB via the A- and B^{0+}-system. Still, the presence of 5-OPase or other enzymes of the γ-glutamyl cycle apart from GGT have not been investigated by direct immunohistochemical methods at the BBB, although of 5-OPase activity has already been shown at the choroid plexus, locus of the barrier between blood and liquor cerebrospinalis [10] and more recently in brain capillaries and BMECs [11].

The present study was conducted to further elucidate the role of GGT and to support the idea of the γ-glutamyl cycle at the BBB by investigating the expression of 5-OPase in porcine brain. An antiserum raised against 5-OPase from porcine kidney supports the finding that 5-OPase is localized in brain microcapillaries, endothelial cells as well as in pericytes but not in peripheral microvessels.

2. Materials and methods

2.1. Enzyme purification and preparation of antibodies

5-OPase was partially purified from porcine kidney according to [12] with some minor modifications. After size exclusion chromatography, the sample containing 5-OPase activity was subjected to preparative SDS-PAGE with subsequent Coomassie-staining. The 5-OPase band (140 kDa) was cut out and the protein recovered by electroelution and precipitation [13]. Rabbit antibodies were generated by Eurogentec (Seraing, Belgium). Isolation of IgGs from the antiserum was performed according to Harboe and Ingild [14]. The IgGs recognized a 140 kDa band in crude kidney lysate together with a 50 kDa signal caused by cross-reactivity with porcine IgGs. Cross-reactive antibodies were removed by incubation of the antibody-solution with powdered nitrocellulose coated with porcine IgGs [15]. Non-bound immunoglobulins merely contained 5-OPase-specific antibodies and were used for immunohistochemical studies.

2.2. Immunohistochemical staining

Small tissue cubes from porcine cerebrum, cerebellum, kidney and skeletal muscle were fixed in 4% paraformaldehyde/PBS. For 5-OPase-specific staining, paraffin sections (10 μm) were used. After removal of paraffin, defatting, permeabilization and rehydration, proteins were denatured with SDS in PBS (1 g/100 ml). SDS was removed by three washes with PBS. Blocking was carried out with bovine serum albumin in PBS (3 g/100 ml). The buffered primary antibody solution contained 400 μg/ml affinity-purified rabbit anti-5-OPase IgG and additionally 1.4 mg/ml heat denaturated IgGs from pig serum. Secondary antibody binding was performed with a 1:200 dilution of biotin-labelled goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) in PBS with 1 g/100 ml bovine serum albumin. The sections were then stained with a streptavidin-phycoerythrin staining kit (Amersham Buchler, Braunschweig) as recommended by the manufacturer. Reference sec-

*Corresponding author. Fax: (49) (6151) 165399.
E-mail: swolf@pop.tu-darmstadt.de

Abbreviations: BBB, blood-brain barrier; BMEC, brain microvessel endothelial cell; GGT, γ-glutamyl transpeptidase; 5-OPase, 5-oxo-L-prolinase

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tions were treated with preimmune serum diluted to approximately 3.7 mg IgG/ml with PBS containing 0.4 mg/ml bovine serum albumin.

Cryo-sections (10 μm) were used in experiments with the laminin antiserum. Laminin-specific staining was carried out with an antiserum (rabbit) generated against mouse laminin with cross-reactivity towards porcine laminin (R. Deutzmann, Regensburg). IgG concentration of the laminin antiserum was 10 μg/ml, PBS containing 0.4 mg bovine serum albumin/ml during the primary antibody binding. In these cases, secondary antibody binding was performed with a Texas-Red-labelled goat anti-rabbit IgG (Vector Lab., Burlingame), 15 μg/ml in PBS containing 1 g/100 ml bovine serum albumin.

3. Results

In order to analyze 5-OPase distribution in porcine cerebrum and cerebellum, we prepared tissue sections from formalin-fixed, paraffin-embedded porcine brain. Since the antibody was raised against SDS-treated 5-OPase, pretreatment of all tissue slices with the detergent was evident to obtain significant staining. Indirect immunofluorescence staining showed 5-OPase localization especially in smaller vessels of brain cortex and cerebellum, whereas larger vessels remain unstained (Fig. 1A, B). The inner diameter of 5-OPase positive vessels ranges from 3.0 to 5.2 μm, with an average of 4.1 ± 0.9 μm. A narrow band of staining surrounds the microvessels. In some cases, cross and longitudinal sections of capillaries showed an asymmetrical staining. Control sections treated with preimmune serum remain unstained (Fig. 1C). Comparative studies with an anti-laminin antiserum showed an overall staining of capillaries, as well as larger vessels, due to the occurrence of laminin in all basement membranes (Fig. 1D).

When examining porcine kidney, strongest immunoreactivity against 5-OPase was observed in proximal tubules (Fig. 1E), whereas glomeruli and blood vessels remained unstained. In order to demonstrate that peripheral microvessels lack 5-OPase, slides of skeletal muscle were also investigated. As shown in Fig. 1F, only a diffuse background staining was obtained.

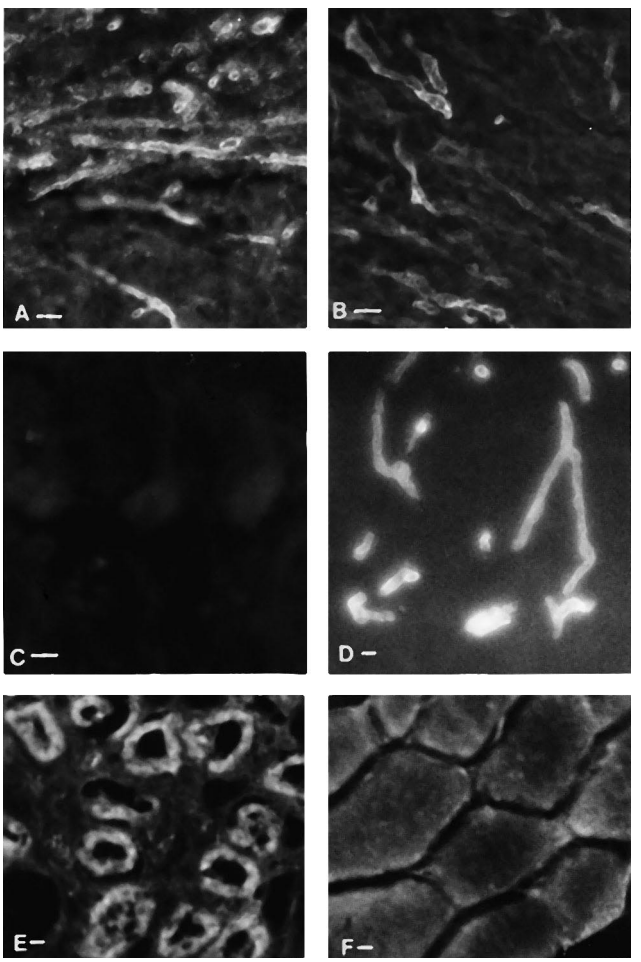

Fig. 1. A: microvessels of pig cerebellum stained with the purified anti-5-OPase antibody. B: anti-5-OPase positive microvessels of pig cortex. C: control sections of pig cerebellum treated with the preimmune serum showed a weak background staining of Purkinje neurons. D: immunoreactivity for anti-laminin (mouse) demonstrates the overall staining of all blood vessels, even larger than microcapillaries. E: tissue section of pig kidney stained with the affinity purified anti-5-OPase antibody, displaying illuminated tubular structures but not glomerular or any other blood vessels. F: the lack of any 5-OPase-specific staining in skeletal muscle emphasizes that the enzyme is not expressed by endothelial cells of peripheral blood vessels. The staining procedure was carried out as given in Section 2. All micrographs were taken from 10 μm sections of paraffin-embedded tissue (5-OPase) or cryosections (laminin) from porcine brain. 1 Bar = 10 μm.

4. Discussion

Since GGT has been detected in brain capillaries [16], the enzyme was regarded as a BBB marker enzyme. In peripheral tissues, such as kidney, GGT is expressed by the epithelium of proximal tubules [17] but not in blood vessels. Orlowski and coworkers [4] postulated that GGT in brain tissues may be involved in amino acid transport via the γ-glutamyl cycle. However, GGT participates also in detoxicative pathways (mercapturic acid pathway and leukotriene metabolism). Consequently, the sole fact that GGT occurs in brain capillaries does not demonstrate the occurence of the γ-glutamyl cycle at the BBB.

To prove that the γ-glutamyl cycle is present in brain microcapillaries, we have chosen another enzyme of this pathway, 5-OPase. The immunohistochemical study in pig tissues presented here showed vessel-specific staining only in cortex and cerebellum. Calculated from the inner diameter, those vessels can be regarded as capillaries. In particular cases, cross and longitudinal sections of capillaries seem to have asymmetrical staining pattern that could be due to the expression of the enzyme in pericytes.

Our data therefore support the amino acid transport hypothesis via the γ-glutamyl cycle at the BBB. We also conclude that 5-OPase can be regarded as a new BBB marker, due to its absence in peripheral vessels of skeletal muscle and kidney. Even though the localization of 5-OPase in pericytes remains to be elucidated by electron microcopy, the staining pattern at the light microscopic level seems to be similar to that of GGT in porcine brain [5].

Although GGT has been under investigation at the BBB for several decades, the localization of the enzyme in brain microvessel constituting cell types was under controversial discussion, as well as the occurence on luminal or abluminal membranes [5,18,19]. More recently, species differences have been demonstrated for both brain barrier tissues, brain capillaries and plexus chorioideus [8,20]. Interestingly, Zlokovic et al. could not find any GGT-mediated degradation of glutathione on the luminal side of perfused brain microvessels [21], which

gives indirect evidence for a non-luminal orientation of the enzyme.

Lee et al. [9] proposed a model for the action of the γ-glutamyl cycle by following the idea of a luminal localization of GGT. They hypothesized that the enzyme uses blood-borne substrates to produce γ-glutamyl amino acids which subsequently enter the brain endothelium and are converted into free amino acids and 5-oxo-L-proline. As shown in their study, this metabolite is able to activate the B^{0+}- and A-system for amino acid transport at the abluminal membrane of endothelial cells. 5-OPase therefore plays a crucial role within this pathway at the BBB by degrading the transport-stimulating molecule.

In view of the data presented in our study, together with the abluminal orientation of GGT in porcine brain microvessels [5,6], the regulation of retrograde transport of neurotransmitter amino acids by the γ-glutamyl cycle should also be considered.

However, 5-OPase activity in the brain capillaries may not only be due to the occurence of the γ-glutamyl cycle in this tissue. Cerebral 5-oxo-L-proline is additionally produced e.g. by the degradation of certain neuropeptides [22] and must be removed from the brain due to its neuromodulatory and toxic properties [23,24].

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