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Pepsin pretreatment allows collagen IV immunostaining of blood vessels in adult mouse brain

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Abstract

While the brain vasculature can be imaged with many methods, immunohistochemistry has distinct advantages due to its simplicity and applicability to archival tissue. However, immunohistochemical staining of the murine brain vasculature in aldehyde fixed tissue has proven elusive and inconsistent using current protocols. Here we investigated whether antigen retrieval methods could improve vascular staining in the adult mouse brain. We found that pepsin digestion prior to immunostaining unmasked widespread collagen IV staining of the cerebrovasculature in the adult mouse brain. Pepsin treatment also unmasked widespread vascular staining with laminin, but only marginally improved isolectin B4 staining and did not enhance vascular staining with fibronectin, perlecan or CD146. Collagen IV immunoperoxidase staining was easily combined with cresyl violet counterstaining making it suitable for stereological analyses of both vascular and neuronal parameters in the same tissue section. This method should be widely applicable for labeling the brain vasculature of the mouse in aldehyde fixed tissue from both normal and pathological states.

Keywords: Adult; Antigen retrieval; Blood vessels; Brain; Collagen IV; Immunohistochemistry; Mouse; Pepsin

1. Introduction

A variety of methods have been utilized to label the brain vasculature in histological sections of experimental animals as well as humans. Vessels have been identified using standard histological stains such as silver (Gallyas, 1970) or toluidine blue (Manoonkitiwongsa et al., 2001), histochemical assays for enzymes enriched in blood vessels (Bell and Scarrow, 1984; Lee et al., 2005) as well as immunohistochemistry for vascular associated antigens. In animals, the cerebrovasculature has also been labeled *in vivo* by perfusion with photographic emulsions (Boero et al., 1999; D'Andrea, 2004), India ink (Shimada et al., 1992) or lectins (Xu et al., 2004).

While all these methods have been used with success, immunohistochemistry has many advantages including its simplicity, the wide range of antigens available, its applicability to archival tissue and its suitability for quantification by stereological methods. A variety of antigens expressed on either endothelial cells or the vascular basement membrane have been used including laminin (Eriksdotter-Nilsson et al., 1986), fibronectin (Krum et al., 1991), von Willebrand factor (Theilen and Kushonsky, 1992), collagen IV (Gidday et al., 2005) and the heparan sulfate proteoglycan (HSPG) perlecan (Bailey et al., 2004; Buée et al., 1994; Chen et al., 2005; Gidday et al., 2005). All work well in embryonic or neonatal mouse and rat brain. They also generally work well on frozen sections of

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adult brain (Mori et al., 1992). However, most blood vessels in paraformaldehye fixed tissue from adult rodent brain stain poorly or not at all for these antigens (Jucker et al., 1992; Mori et al., 1992), the only major exception being that vessels in or bordering the sites of cerebral lesions generally stain well even in fixed tissue (Jucker et al., 1992; Krum et al., 1991; Mori et al., 1992; Szabo and Kalman, 2004).

Why tissue fixation should differentially effect vascular staining in embryonic/neonatal versus adult rodent brain is not known. Interestingly, there has been little investigation of whether antigen retrieval methods that are commonly utilized in human postmortem tissue might improve vascular staining in adult rodent brain and in particular whether such methods might overcome the general problem of staining normal blood vessels in fixed tissue from adult rodent brain. Here we investigated whether several commonly used antigen retrieval methods could improve staining of the vasculature in adult mouse brain. We show that pepsin digestion prior to immunostaining with collagen IV results in widespread vascular staining in adult brain. When combined with cresyl violet staining this method produces sections that are suitable for collecting stereological data sets on both vascular and neuronal parameters from the same section.

2. Materials and methods

2.1. Animals

Six-month-old male C57Bl/6J mice (stock name C57Bl/6J; stock number 000664) and TIE2-GFP mice (stock name B6.Cg-Tg(TIE2GFP)287Sato/1J; stock number 004659) were purchased from Jackson Laboratories (Bar Harbor, MA, USA). Animals were housed under controlled environmental conditions on 12 h light/dark cycles with *ad libitum* access to food and water. All protocols were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee and were in conformance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals".

2.2. Tissue processing

Adult mice were anaesthetized with 150 mg/kg ketamine and 30 mg/kg xylazine and then sacrificed by transcardial perfusion with cold 1% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.4 for 1 min, followed by cold 4% paraformaldehyde in PBS for 10 min. After perfusion, brains were removed and postfixed in the 4% paraformaldehyde for 48 h and then transferred to 0.1 M PBS, and stored at 4 °C until sectioning. Fifty μm-thick coronal sections were cut using a Vibratome. In all studies where different tissue processing treatments were compared, adjacent sections from the same animal were always used for each of the treatments being compared.

Embryos were isolated from timed pregnant female mice with the day a vaginal plug was observed considered as E0.5. Following euthanasia of the mother with CO_2, embryos were isolated, fixed in 4% paraformaldehyde in 0.1 M PBS pH 7.4 and cut into 40 μm-thick sections on a Vibratome.

2.3. Antigen retrieval

Pepsin digestion was carried out on free-floating sections using methods similar to those described in Fukaya and Watanabe (2000). Prior to pepsin treatment, sections were incubated in distilled water for 5 min at 37 °C and then transferred to 1 mg/ml pepsin (Dako, Carpinteria, CA) in 0.2N HCl. Sections were incubated in the pepsin solution at 37 °C for 10 min. After washing in PBS for 15 min at 27 °C followed by three 10 min washes at room temperature, sections were processed for immunohistochemistry as described below.

For antigen retrieval using citrate buffer pH 3.0, sections were incubated in 10 mM sodium citrate at 37 °C for 30 min, allowed to cool to room temperature for 20 min and then processed for immunohistochemistry. Antigen retrieval using sodium citrate pH 6.0 (Antigen Unmasking Solution, H-3300; Vector Laboratories) was carried out according to manufacturer's instructions. Briefly, sections were incubated in distilled water for 5 min followed by incubation in unmasking solution at 95 °C for 1 min. Sections were cooled to room temperature and washed in PBS for 5 min prior to processing for immunohistochemistry.

2.4. Histology and immunohistochemistry

Immunohistochemistry was performed on free-floating sections. The primary antibodies used were a rabbit polyclonal anti-collagen IV (1:500; Chemicon, Temecula, CA, USA), a rabbit polyclonal anti-laminin (1:100; Sigma, St. Louis, MO, USA), a rabbit polyclonal anti-fibronectin (1:400; Sigma), a mouse monoclonal anti-CD146 (1:300; Chemicon), a rat monoclonal anti-perlecan (1:500; Neomarkers, Fremont, CA, USA) and a rabbit anti-GFP (1:1000; Invitrogen, Carlsbad, CA, USA). Sections incubated without primary antibody served as controls. Sections were blocked with Tris-buffered saline (TBS; 50 mM Tris–HCl, 0.15 M NaCl, pH 7.6, 0.15 M NaCl)/0.1% Triton X-100/5% goat serum (TBS-TGS) for 1.5 h and the primary antibody was applied overnight in TBS-TGS at room temperature. Following washing in PBS for 1 h, immunofluorescence staining was detected by incubation with species specific Alexa Fluor secondary antibody conjugates (1:400; Molecular Probes, Burlingame CA, USA) for 2 h in TBS-TGS. Nuclei were counterstained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI). After washing in PBS, sections were mounted on slides using Gel/Mount (Biomeda, Foster City, CA, USA). Staining with *Griffonia simplicifolia* isolectin B4 was performed as described in Wen et al. (2005) except that Vibratome sections were used.

For immunoperoxidase staining with collagen IV, sections were pretreated with 10% methanol/1% hydrogen peroxide in PBS for 10 min. After a wash in PBS, primary antibody was applied as described above followed by a goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. Staining was visualized using 3,3′-diaminobenzidine (DAB) in 50 mM Tris-imidazole buffer (pH 7.6). Sections were mounted on slides, dried overnight and counterstained with 0.5% cresyl violet for 6 min followed by dehydration with a graded series of ethanol solutions (70, 85, 90, 100% for 2 min each).