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Pepsin Pretreatment Allows Collagen IV Immunostaining of Blood Vessels in Adult Mouse Brain

Sonia Franciosi^{a,d,e}, Rita De Gasperi^{a,e}, Dara L. Dickstein^b, Daniel F. English^{a,d}, Anne B. Rocher^b, William G.M. Janssen^b, Daniel Christoffel^b, Miguel A. Gama Sosa^{a,e}, Patrick R. Hof^b, Joseph D. Buxbaum^{a,b,c,d}, and Gregory A. Elder^{a,e}

^aDepartment of Psychiatry, Mount Sinai School of Medicine, New York, NY 10029 USA

^bDepartment of Neuroscience, Mount Sinai School of Medicine, New York, NY 10029 USA

^cDepartment of Human Genetics, Mount Sinai School of Medicine, New York, NY 10029 USA

^dDepartment of Laboratory of Molecular Neuropsychiatry, Mount Sinai School of Medicine, New York, NY 10029 USA

^eResearch and Development Service, James J. Peters Department of Veterans Affairs Medical Center, Bronx, NY 10468 USA

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

Corresponding Author: Gregory A. Elder Department of Psychiatry, Box 1230, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA James J. Peters Department of Veterans Affairs Medical Center, Research and Development Service (3F22), 130 West Kingsbridge Road, Bronx, NY, 10468, USA Email: gregory.elder@mssm.edu

Sonia Franciosi, Departments of Psychiatry and Laboratory of Molecular Neuropsychiatry, Box 1668, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

Rita De Gasperi, Department of Psychiatry, Box 1230, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA; James J. Peters Department of Veterans Affairs Medical Center, Research and Development Service (3F20), 130 West Kingsbridge Road, Bronx, NY, 10468, USA

Dara L. Dickstein, Department of Neuroscience, Box 1065, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

Daniel F. English, Departments of Psychiatry and Laboratory of Molecular Neuropsychiatry, Box 1668, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

Anne B. Rocher, Department of Neuroscience, Box 1065, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

William Janssen, Department of Neuroscience, Box 1065, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

Daniel Christoffel, Department of Neuroscience, Box 1065, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

Miguel A. Gama Sosa, Department of Psychiatry, Box 1230, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA; James J. Peters Department of Veterans Affairs Medical Center, Research and Development Service (3F20), 130 West Kingsbridge Road, Bronx, NY, 10468, USA

Patrick R. Hof, Department of Neuroscience, Box 1065, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

Joseph D. Buxbaum, Departments of Psychiatry, Neuroscience, Human Genetics, Laboratory of Molecular Neuropsychiatry, Box 1668, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

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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 (Lee et al., 2005; Bell et al., 1984) 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 et al., 1992), collagen IV (Gidday et al., 2005) and the heparin sulfate proteoglycan (HSPG) perlecan (Chen et al., 2005; Gidday et al., 2005; Bailey et al., 2004; Buée et al., 1994). All work well in embryonic or neonatal mouse and rat brain. They also generally work well on frozen sections of adult brain (Mori et al., 1992). However, most blood vessels in paraformaldehyde fixed tissue from adult rodent brain stain poorly or not at all for these antigens (Mori et al., 1992; Jucker 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 (Krum et al., 1991; Jucker et al., 1992; Mori et al., 1992; Szabo and Kalman, 2004).

Why tissue fixation should differentially effect vascular staining in embryonic/neonatal vs. 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 hr 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 hrs and then transferred to 0.1M 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 CO₂, 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 0.2 N HCl. Sections were incubated in the pepsin solution at 37°C for 10 minutes. 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 minutes, 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 (1:1,000; 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 hrs and the primary antibody was applied overnight in TBS-TGS at room temperature. Following washing in PBS for 1 hr, immunofluorescence staining was detected by incubation with species specific Alexa Fluor secondary antibody conjugates (1:400, Molecular Probes, Burlingame CA, USA) for 2 hrs 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 two hours. 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). Slides were then treated with Americlear (Fisher, Tustin, CA, USA) for 2 min, followed by xylene for 10 min and coverslipped with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI, USA).

Sections were photographed on a Zeiss Apotome microscope using the AxioVision Release 4.3 program (Zeiss, Thornwood, NY, USA) or a Nikon Eclipse E400 connected to a DXC-390 CCD camera (Nikon, Melville, NY, USA). Digital images were color balanced using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

3. Results

Blood vessels in embryonic and early postnatal rodent brain can be readily stained under a variety of fixation conditions either by immunohistochemistry or with markers such as lectins. By contrast, our experience parallels that of others (Jucker et al., 1992; Krum et al., 1991; Szabo et al., 2004) that these same methods mark blood vessels in adult mouse brain poorly and inconsistently. For example, Figure 1 shows collagen IV and isolectin B4 staining on Triton X-100-permeabilized sections of neocortex from a six-month C57Bl/6J mouse that had been perfused with 4% paraformaldehyde and the brain postfixed for 48 hrs in the same fixative. As shown in Figure 1A and B, no collagen IV or isolectin B4 labeled vessels were apparent. By contrast, Figure 1D and E show examples of collagen IV and isolectin B4 staining on sections of E15 mouse neocortex that had been immersion fixed for 48 hrs in 4% paraformaldehyde. The vessels throughout the sections are prominently stained with both markers. We have had very similar experience with a variety of other antigens including laminin, fibronectin and perlecan, in each case finding minimal to no staining of the cerebrovasculature in adult mice despite widespread labeling of embryonic vessels by each marker (data not shown).

The differing staining characteristics of embryonic and adult cerebral blood vessels could reflect differences in antigen expression levels or, alternatively, the antigens could be present but less exposed in adult vessels. Because antigen inaccessibility offered one explanation for the difficulty of immunostaining cerebral vessels in adult mouse brain, we determined whether several commonly utilized antigen retrieval methods might improve vascular labeling.

High temperature treatment has been one of the most successfully used antigen recovery methods (Shi et al., 1997; Jiao et al., 1999). Therefore we initially tried improving collagen IV immunostaining by heating sections to 95° C for 1 min in a commercially available citrate buffered (pH 6.0) antigen retrieval solution (Antigen Unmasking Solution, Vector Laboratories). However, this treatment led to no improvement in vascular staining with collagen IV (Figure 2C). Since some antigens are most effectively unmasked at low pH (Shi et al., 1997), we also tried treating sections with 10 mM citric acid (pH 3.0), another commonly used antigen retrieval method (Shi et al., 1997). However, we again found no improvement in collagen IV immunolabeling of cerebral blood vessels (Figure 2E).

Enzymatic treatments have also been effectively utilized to unmask certain antigens (MacIntyre, 2001). Pepsin treatment in particular has been reported to improve detection of

many antigens (Mori et al., 1992;Krum et al., 1991). We therefore determined whether digestion with 1 mg/ml pepsin in 0.2 N HCl for 10 mins at 37° C would improve subsequent collagen IV immunostaining. As shown in Figure 2G pepsin pretreatment led to widespread vascular labeling throughout adult mouse brain. We have also found that antigen retrieval with pepsin is as effective in 0.5 M acetic acid as it is in 0.2 N HCl (data not shown).

As an indicator of the efficiency of vascular labeling with collagen IV, we compared collagen IV immunostaining with vascular labeling in the Tie2-GFP mouse, which express a green fluorescent protein (GFP) under the control of the endothelial cell specific Tie2 promoter (Motoike et al., 2000). Initially, we tried to directly compare collagen IV with GFP labeling by double staining Tie2-GFP tissue for both antigens. However, the pepsin treatment completely abolished GFP immunostaining. Since a direct comparison was not possible, we compared collagen IV staining in pepsin treated sections to adjacent non-treated sections stained for GFP. As shown in Figure 3, the patterns of collagen IV (Figure 3A) and GFP (Figure 3B) staining appeared quite similar, arguing that collagen IV immunolabeling likely detects a population of vessels as extensive as those labeled with GFP in the Tie2-GFP mouse.

We next examined the utility of pepsin antigen retrieval for unmasking other vascular antigens as well as isolectin B4 staining. Representative examples of immunohistochemical staining for laminin and isolectin B4 with or without prior pepsin digestion are shown in Figure 4. Pepsin treatment also resulted in vascular staining with laminin (Figure 4B), and isolectin B4 (Figure 4D). The laminin staining appeared as extensive as that with collagen IV although neuronal laminin staining also became apparent after pepsin treatment (arrows, Figure 4B). Labeling with isolectin B4 was much less complete and did not stain the entire vasculature (Figure 4D). Pepsin treatment did not enhance vascular staining for fibronectin, perlecan or CD146 (data not shown).

Stereologic data sets on vascular parameters are commonly collected in immunoperoxidase stained material. As shown in figures 5A and 5C without pepsin treatment no collagen IV immunoperoxidase stained vessels were visible in adult mouse brain while following pepsin treatment collagen IV stained vessels became readily apparent (Figures 5B, D, E, F). When estimates of neuronal parameters are assessed in the same tissue, they are commonly obtained in adjacent sections stained with cresyl violet. However, in some situations such when tissue is limiting, it would be advantageous to obtain stereologic assessments of both vascular and non-vascular parameters in the same tissue section. We therefore tested whether the pepsin treatment would affect Nissl staining with cresyl violet. As shown in Figure 5, a cresyl violet stain applied after collagen IV immunostaining was uniform and stained neuronal as well as non-neuronal cells throughout the section. Thus collagen IV immunostaining combined with cresyl violet counterstaining is an effective method for labeling both vascular and non-vascular elements in the same section.

4. Discussion

Aldehyde-based fixatives react with basic amino acids principally lysine and arginine, generating intra- and intermolecular interactions that crosslink and stabilize proteins *in situ* (Shi et al., 1997;MacIntyre, 2001). Although beneficial for preserving cellular structure, these interactions may alter antigenic epitopes or inhibit antibody access, thereby interfering with immunohistochemical procedures. Antigen retrieval methods developed largely as a response to the problems encountered when immunohistochemical staining procedures were applied to paraffin-embedded human archival tissue. Many variations now exist, with the most common involving pretreatment of sections with heat, high or low pH buffers, or proteolytic enzymes (Shi et al., 1997;MacIntyre, 2001).

Here we show that pepsin digestion prior to immunostaining unmasks widespread collagen IV staining of the cerebrovasculature in paraformaldehyde fixed sections of adult mouse brain (Figure 2G). Whether performed in 0.2 N HCl or 0.5M acetic acid, the collagen IV staining appeared to stain the entire cerebral vasculature including vessels of all size ranges and appeared co-extensive with the GFP labeling of cerebral vessels in the Tie2-GFP mouse (Figure 3). While we did not undertake an exhaustive examination of the various antigen retrieval methods, we did find that neither high temperature (Figure 2C) nor low pH treatments (Figure 2E) (Shi et al. 1997) permitted recovery of collagen IV staining. In addition to collagen IV, pepsin treatment unmasked vascular staining with laminin (Figure 4B) as has also been reported by others (Krum et al., 1991; Mori et al., 1992). Pepsin treatment in addition partially improved isolectin B4 staining, although vascular labeling was incomplete (Figure 4D). Vascular staining with isolectin B4 in adult rat brain has also been reported to improve if it follows a DNA denaturation step (Ernst and Christie, 2006).

By contrast, pepsin treatment did not enhance vascular staining for fibronectin, perlecan or CD146, although it unmasked neuronal fibronectin staining (data not shown). The failure to unmask perlecan staining is curious, since antibodies to perlecan readily stain cerebral blood vessels in paraffin-embedded sections of adult human brain, which are typically pepsin digested (Buée et al., 1997). We are however unaware of any published studies reporting perlecan staining of normal cerebral blood vessels in adult mouse brain. This variability could reflect additional technical differences between tissue processing protocols typically used in human and mouse studies. Alternatively, it may indicate structural differences between mouse and human vessels or the amount of perlecan in the vascular basement membrane in different species.

Why immature brain vessels stain so readily with collagen IV as well as other antigens without antigen retrieval is not known but may reflect differences in the tightness of the gliovascular junctions. Indeed one of the major structural differences between immature and mature vessels in brain is the large perivascular spaces present in immature vessels that separate the outer glial basal lamina and the inner vascular one (Caley and Maxwell, 1970; Szabo and Kalman, 2004). In the rat, after about P10 the glial and vascular basal laminae fuse and the extravascular spaces are largely eliminated. Interestingly, this corresponds to the developmental time point when vascular staining with laminin is lost in paraformaldehyde-fixed tissue stained without the benefit of pepsin treatment (Krum et al., 1991; Szabo and Kalman, 2004).

The relative tightness of the gliovascular junctions may also explain the immunostaining of blood vessels in pathological states. For example, following stab injuries, cerebral blood vessels near the lesion stain prominently with laminin without the need for antigen retrieval (Mori et al., 1992). This staining is progressively lost as vessels become more distant from the lesion and decreases in vessels in the lesion area during the repair process. Szabo and Kalman (2004) have shown that both the degree of laminin staining as a function of distance as well as the loss of laminin staining during recovery correlates strongly with the tightness of the gliovascular junctions. Areas of cerebral ischemia constitute another example where in adult rodent brain vascular staining with collagen IV occurs in fixed tissue without antigen retrieval (Jucker et al., 1992) and interestingly breakdown of gliovascular junctions is known to be one of the earliest events morphologically following ischemia (Wagner et al., 1997).

How pepsin digestion unmasks collagen IV immunostaining is uncertain although among protease treatments pepsin appears to have some specificity in that collagen IV staining is not unmasked by proteinase K treatment (Davis et al., 2004). Proteolytic digestion presumably disrupts protein cross links that form during fixation and in the case of laminin staining it has been suggested that pepsin may disrupt cross links between laminin and collagen IV (Mauro et al., 1984) although this mechanism remains speculative.

Yet, whatever the basis for its beneficial effects, here we show that pepsin digestion effectively unmasks collagen IV immunostaining of the vasculature in adult mouse brain. The staining that results was widespread and was easily combined with cresyl violet counterstaining (Figure 5). This method should be widely applicable for labeling the brain vasculature of adult mice in both normal and pathological states.

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Figure 1.

Cerebrovascular staining in adult and embryonic murine brain using standard immunohistochemistry without antigen retrieval. Representative photomicrographs from the cortex of an adult C57Bl6/J mouse shows no cerebrovascular staining with (A) collagen IV or (B) isolectin B4. Arrows indicate vascular profiles that are unstained. Nuclear counterstaining of panel (B) is shown in (C) with the cortical layers indicated. In contrast, representative photomicrographs of developing neocortex from E15 mouse brain show that embryonic tissue can be readily double labeled with (D) collagen IV and (E) isolectin B4 using standard immunohistochemical techniques. Nuclear counterstaining with DAPI is shown in panel (F) where the cortical plate (CP) is indicated. Scale bar: 50 μ M.

Figure 2.

Antigen retrieval results in labeling of the cerebrovasculature in adult mouse brain. Representative photomicrographs of neocortical tissue from adult C57Bl6/J mouse brain showing that (A) without antigen retrieval (AR) or following (C) antigen retrieval with citrate buffer pH 6 or (E) citrate buffer pH 3 there is no cerebrovascular staining of collagen IV. However, pepsin antigen retrieval (G) resulted in widespread collagen IV staining. Nuclear counterstaining with DAPI is shown in the lower panels for the untreated (B) and tissue exposed to the different antigen retrieval techniques (D, F, H). Scale bar: 50 μ M.

Figure 3.

Collagen IV labeling of cerebral vessels with pepsin antigen retrieval compared to GFP stained vessels in the Tie2-GFP mouse. (A) Vascular staining with collagen IV following pepsin antigen retrieval in the Tie2-GFP mouse. (B) Typical GFP-immunostained cerebral vessels in an adjacent section. Note the similar pattern and extent of GFP vascular labeling to the collagen IV labeling in panel (A). (C) DAPI nuclear staining of the section stained in panel (A). Scale bar: 50 μ M.

Figure 4.

Pepsin antigen retrieval leads to cerebrovascular labeling with other vascular antigens. Representative photomicrographs are shown of cerebral vessel staining without (A, C) and with prior pepsin antigen retrieval (AR) for (B) laminin and (D) isolectin B4. Vascular staining of laminin became apparent following AR. However, neuronal laminin staining was also unmasked (indicated by arrows). Isolectin B4 staining improved with pepsin AR, but was incomplete. Scale bar: 50 μ M.

Figure 5.

Pepsin antigen retrieval also allows visualization of cerebral vessels with immunoperoxidase staining and does not interfere with cresyl violet counterstaining. Representative photomicrographs are shown from the hippocampal CA1 field and corpus callosum (A, B), entorhinal cortex (C, D), neocortex (E), and dentate gyrus (F) from a C57Bl6/J mouse immunoperoxidase stained for collagen IV without (A, C) or following (B, D, E, F) pepsin antigen retrieval. Sections were counterstained with cresyl violet. Note the lack of vascular staining without antigen retrieval although non-specific peroxidase staining can be seen at the edge of the section in panel C. Following antigen retrieval peroxidase staining of the cerebrovasculature is apparent as well as Nissl staining of the surrounding neuropil. Scale bar: 40 μ M.