Isolectin-IB4 as a vascular stain for the study of adult neurogenesis

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Abstract

It has been proposed that new neurons in the adult brain might preferentially develop in the immediate vicinity of existing vasculature, an area that has been referred to as a “vascular niche”. The most common method for identifying neurons that arise as the result of mitotic activity is to label them with the thymidine analogue bromodeoxyuridine (BrdU), and a nuclear marker for mature neurons (NeuN). Unfortunately, commercially available forms of NeuN and the vasculature stain, RECA-1, are incompatible, as both antibodies are raised in the same species. This technical limitation has impeded the analysis of the relationship between new neurons and existing vasculature. In the present work we show that Isolectin-IB4, a 114 kDa protein isolated from the seeds of the African legume, Griffonia simplicifolia, can be used to identify vasculature in conjunction with both NeuN and BrdU immunohistochemistry. Isolectin-IB4 serves as an easy to use and robust marker for obtaining spatial information regarding the proximity of new neurons to vasculature in the mammalian brain.

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1. Introduction

Adult neurogenesis refers to a process that results in the formation of new neurons in the mature brain, and in many ways can be viewed as an extension of the process of neurogenesis that is normally associated with the patterning and development of the nervous system. It has been well documented that new neurons can originate in both the subgranular zone (SGZ) of the dentate gyrus and in the subependymal zone (SEZ) of the lateral ventricles (Alvarez-Buylla and Lim, 2004). Both of these areas are tightly bordered with blood-filled vessels that naturally serve to provide nutrients and facilitate gas exchange with the surrounding tissue. It follows then, that if blood vessels border the SGZ and SEZ, and that the blood vessels are responsible for providing nutrients to the surrounding tissue, that new cells could preferentially grow in these niches (Palmer et al., 2000). The vascular niche hypothesis proposes that the angiogenic niche that is required for endothelial cell recruitment may also be responsible for stimulating the growth of new neurons (Fabel et al., 2003; Jin et al., 2002; Palmer, 2002; Palmer et al., 2000).

To visualize whether new neurons do indeed grow in vascular niches, one should ideally triple-stain tissue for (1) a neuronal marker such as NeuN, (2) a marker of existing vasculature, and (3) a marker of mitotic activity such as BrdU. Any cell that stains with both BrdU and a mature neuronal marker could then be assessed for its proximity to the vasculature marker. Currently, the marker used to label the surrounding vasculature is rat endothelial cell antigen-1 (RECA-1, Palmer et al., 2000; Palmer, 2002). This marker has the unfortunate co-incidence of being raised in the same animal (mouse) as the most commonly used neuronal marker, NeuN. This means that NeuN and RECA-1 cannot be easily visualized in the same tissue, due to problems with cross-reactions by secondary antibodies. To better understand the relationship between neural vasculature and new neurons, it would be ideal to have a vascular marker that could be used in conjunction with NeuN and BrdU. In this way, one could better quantify neurogenesis near the vasculature instead of being limited to studies of cell proliferation. This is an important consideration as several distinct, and mitotically active,
cell types reside near the vasculature including glia, endothelial, and neuronal cells. Thus it is essential to determine the phenotype of any cells being generated in these regions. Isolectin-IB4 is a 114 kDa protein isolated from the seeds of the African legume, *Griffonia simplicifolia* and as such is known to bind to microglia, group B erythrocytes, perivascular cells, and endothelial cells (Grossmann et al., 2002; Tarpley et al., 2004). Here we demonstrate that Isolectin-IB4 can be used to stain vasculature in the adult brain in conjunction with immunohistochemical markers for mitotic activity and mature neurons.

2. Materials and methods

2.1. Subjects

Five adult male Sprague–Dawley rats (250–300 g) were obtained from UBC animal care services and individually housed for a period of 42 days while kept on a standard 12 h light/dark cycle. Food and water were available ad libitum during this period. All animals were treated according to the guidelines of the Canadian Council on Animal Care.

2.2. BrdU injections and tissue preparation

To label newly synthesized DNA, animals were injected once with 5-bromo-2-deoxyuridine (BrdU, 200 mg/kg on day 1). After a period of 42 days, animals were given an overdose of sodium pentobarbital and then injected through the heart with 0.9% saline to clear blood from the vasculature, followed by 4% formaldehyde solution to fix the tissue. Brains were then rapidly removed and stored in 4% formaldehyde at 4°C for 2 days to further enhance tissue fixation. For cryopreservation, the intact brain was transferred to a 30% sucrose solution until saturated. Brains were then sliced in 40 μm coronal sections using a cryostat at −15°C (Microm, 500 OM, Heidelberg, Germany). Individual whole brain sections were stored in series in well-plates containing Tris buffered saline (TBS, trizma hydrochloride, trizma base, sodium hydrochloride; Fisher Scientific, Nepean, Ont.) at 4°C (Eadie et al., 2005; Farmer et al., 2004).

2.3. Immunohistochemistry

Tissue sections (never more than 6 slices/well) were first heated in a formamide solution for 2 h at 65°C to denature DNA (50% formamide, 40% dH2O, 10% 20× Saline-SodiumCitrate [SSC: 175.3 g NaCl; 88.2 g sodium citrate in 800 ml dH2O]). Tissues were then washed once for 5 min in 2× SSC. Next, to break any remaining hydrogen bonds between the nitrogenous bases of nucleic acids, slices were heated at 37°C for 30 min in 2N HCl. To regain pH balance, tissue sections were placed in a borate solution (80% dH2O, 20% borate stock solution) at pH 8.5. To decrease background staining, tissues were washed for 15 min in TBS (pH 7.5). This step was repeated six times and tissue was continuously agitated during each one of these rinses (Stovall Belly Dancer® orbital shaker, Greensboro, NC). Next, to block nonspecific binding and to permeabilize cell membranes to allow entry of antibodies, the tissue was saturated with a solution containing 96% TBS, 1% TritonX, and 3% donkey serum (TBS++) before being incubated in primary antibodies against the neuronal nuclear protein NeuN (1:50, mouse anti-NeuN; Chemicon, Temecula, CA) and the thymidine analog, BrdU, a marker of DNA synthesis (1:200, rat anti-BrdU; Serotec, Raleigh, NC). The vasculature marker Isolectin-IB4, was added at the same time as the anti-NeuN and anti-BrdU primary antibodies. Isolectin-IB4 was purchased with an Alexa 488 dye conjugate and did not require secondary antibody treatment (Molecular Probes, Eugene, OR; RECA-1, a ubiquitous marker of endothelial cells (1:25, mouse anti-RECA-1; Serotec, Raleigh, NC). The vasculature marker Isolectin-IB4 was purchased with an Alexa 488 dye conjugate and did not require secondary antibody treatment (Molecular Probes, Eugene, OR; RECA-1, a ubiquitous marker of endothelial cells (1:25, mouse anti-RECA-1; Serotec, Raleigh, NC). We prepared a 1 mM solution of Calcium chloride dihydrate (Fisher Scientific, Nepean, Ont.) and added 1 mL of this solution to 10 mg of Isolectin-IB4. This solution was then added to mixtures at a concentration of 1:100. Tissues were incubated for 60 h at 4°C. NeuN can be a difficult antibody to work with, however, we find that the following staining protocol reliably results.
Fig. 2. Isolectin-IB4 can be used in conjunction with both NeuN and BrdU. (A) Representative example of a triple labeled tissue demonstrating NeuN (green), BrdU (red), and IB4 (blue) in the dentate gyrus. Scale bar: 100 μm. (B1–B3) This is an example of a blood vessel passing through the lateral extent of the granular cell layer of the dentate gyrus. (B1) NeuN, (B2) Isolectin-IB4, (B3) overlay. Scale bar: 25 μm. (C1–C4) New neurons are detectable in the subgranular zone of the dentate gyrus close to the vasculature. Photomicrographs of NeuN (C1), IB4 (C2), BrdU (C3), overlaid (C4) together. Scale bar: 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
in robust staining with this marker of mature neurons. First, when tissue sections are incubated with the primary antibodies, we ensure that the entire surface of the tissue is accessible, and unimpeded by folds. Although tissues are incubated and agitated for 60 h at 4 °C in total, after ~24 h, we readjust and open up any tissue sections that have folded over while being agitated. If the tissue sections are curled up, antibodies may have difficulty accessing the antigens.

After 60 h of incubation in the primary antibodies, we again wash the tissue in TBS (at least three times for 5 min each) to remove excess primary antibody. After this, tissue sections are ready to be stained with secondary antibodies. The secondary antibodies used here included donkey anti-mouse Cy5 and Donkey anti-rat Cy3 (both 1:250, Chemicon, Temecula, CA). Secondary antibodies were incubated, while being agitated, in the dark for 4 h at room temperature. When RECA-1 and Isolectin-IB4 were used together in experiments, they were added simultaneously. In the last step, tissues were mounted on 2% gel coated slides and dried for 1/2 an hour at room temperature before being cover-slipped with Dabco, a free radical scavenger and ImagePro Plus software (Media Cybernetics, San Diego, CA).

3. Results

To demonstrate the ability of Isolectin-IB4 to stain the vasculature, we incubated both Isolectin-IB4 and RECA-1 in the same tissue. As is evident in Fig. 1A, Isolectin-IB4 produced a pattern of staining that is characteristic of that seen with other markers of vasculature endothelial cells. In Fig. 1B, we show that RECA-1, a commonly used endothelial vasculature marker, has a similar staining pattern to that of Isolectin-IB4. In Fig. 1C, an overlay of these dyes in the same tissue sample illustrates how these dyes yield similar patterns of staining with a high degree of overlap. It was difficult to detect any difference between RECA-1 staining or Isolectin-IB4 staining, and observers blinded to the experimental protocol were unable to differentiate the samples based upon staining patterns; however, there was labeling of structures by Isolectin-IB4 that were not labeled by RECA-1. Nevertheless, we found that both markers were capable of detecting the small capillaries in and around the hippocampus, as well as the larger arteries and veins.

To demonstrate the utility of Isolectin-IB4 as an endothelial marker for use in neurogenesis studies, it is essential that this label can be used in conjunction with both NeuN, a marker of mature neurons, and BrdU, a marker of DNA synthesis. As is illustrated in Fig. 2A, Isolectin-IB4 provides a robust labeling of the vasculature when used with antibodies for NeuN and BrdU.

4. Discussion

We have shown that Isolectin-IB4 provides a simple and reliable way to examine the relationship between neurogenic activity and existing vasculature in the adult brain. In particular, we demonstrate that the stain can be used in conjunction with both NeuN and BrdU, two immunohistochemical markers that are commonly used in neurogenesis research. It should be noted that Isolectin-IB4 can bind to microglia, group B erythrocytes, perivascular cells, and endothelial cells, therefore there are some limitations to its use, and the interpretation of data from experiments where these are objects of interest may be less clear. In these situations Isolectin-IB4 should be used in conjunction with high-powered microscopy and specific indicators of non-vascular cells.

The ability of Isolectin-IB4 to clearly identify vasculature in a reliable fashion will make it a useful tool in determining the role the vasculature plays in adult neurogenesis. With the use of Isolectin-IB4 as the vasculature stain, it is now possible to visualize cells in the brain that express neuronal lineage markers, have undergone DNA synthesis, and are in close proximity to the vasculature. With the use of RECA-1, it was only possible to visualize cell proliferation near the vasculature (Kodama et al., 2004; Palmer et al., 2000). The vascular niche hypothesis is one of many ideas put forth to understand why new neurons form only in certain areas of the brain, and this hypothesis may even provide clues as to how cellular migration patterns in the adult brain are derived.

We show here that Isolectin-IB4 is a good vasculature marker to utilize in studies of neurogenic activity in the adult dentate gyrus. This marker provides a point to initiate future studies into the molecular mechanisms that play a role in regulating the synthesis of new neurons in the brain.

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