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Journal of Neuroscience Methods 148 (2005) 26–35

JOURNAL OF NEUROSCIENCE METHODS

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Exposure of nuclear antigens in formalin-fixed, paraffin-embedded necropsy human spinal cord tissue: Detection of NeuN

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Received 29 November 2004; received in revised form 29 March 2005; accepted 30 March 2005

Abstract

Immunohistochemical and immunofluorescence staining approaches are powerful tools for characterization of the endogenous protein expression and subcellular compartmentalization. However, several technical problems hamper identification of low-abundance nuclear proteins in archival formalin-fixed, paraffin-embedded human neural tissue. These include loss of protein antigenicity during tissue fixation and processing, and intrinsic auto-fluorescence associated with the tissue related to its fixation and the presence of lipofuscin. We evaluated several antigen retrieval methods to establish a strategy for detection of neuronal nuclear proteins in human spinal cord formalin-fixed, paraffin-embedded tissue. Thus, using immunostaining of the neuron-specific nuclear protein NeuN as the outcome measure, we found that heating tissue sections in an alkaline pH buffer unmasked protein epitopes most effectively. Moreover, staining by immunohistochemistry with diaminobenzidine tetrahydrochloride chromagen was superior to immunofluorescence labeling, likely due to the signal amplification steps included in the former approach. Auto-fluorescence in the tissue sections can be effectively reduced, but a sufficient fluorescence signal associated with specific antibody labeling could not be detected above this background for NeuN in the nucleus.

Keywords: Nuclear proteins; Antigen retrieval; NeuN; Immunohistochemistry; immunofluorescence; Formalin-fixed; Paraffin-embedded; Necropsy human neural tissue

1. Introduction

The cell is a dynamic structure comprised of multiple subcellular compartments that communicate with each other by movement of proteins and other constituents. Nuclear transport of proteins plays a vital role in communication between the nucleus and other organelles, thus contributing to maintenance of cell viability. Advances in molecular and cellular biology have facilitated research in nuclear transport by providing extensive knowledge of components of nuclear transport machinery (Gorlich, 1998). These include the nuclear pore complexes, transport receptors, soluble proteins such as RanGTP, as well as nuclear localization (NLS) and nuclear export (NES) signals found in proteins that shuttle between the nucleus and the cytoplasm (Kiseleva et al., 2000; Moore, 2001).

In vitro cell models have provided much of our current knowledge about proteins that express functional NLS or NES sequences, but details of nuclear protein localization must also include observations made on the endogenously expressed proteins. As culturing human neural tissue is not generally feasible, investigation of endogenous neuronal nuclear proteins by immunohistochemistry (IHC) must be carried out on fixed or frozen biopsy or necropsy samples prepared either as frozen or paraffin-embedded histological sections. Difficulties that are encountered with fixed-frozen human brain tissue include lack of sample availability (Evers et al., 1998), poor morphological preservation (Onodera et al., 1992), and tissue samples that are not amenable to long-term

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doi:10.1016/j.jneumeth.2005.03.008

storage (Beckstead, 1995; Shi et al., 2001a). These factors restrict investigators ability to conduct retrospective studies (Shi et al., 1993), but some of these limitations can be overcome by working with formalin-fixed, paraffin-embedded necropsy tissues.

Several technical challenges must be resolved to be able to reliably detect low-abundance neuronal nuclear proteins in archival necropsy human neural tissues by IHC. Some of the major difficulties encountered are protein epitopes that become masked during the formalin-fixation and paraffin-embedding process of the tissue, tissue auto-fluorescence, and lipofuscin deposits that increase in abundance with age. Detecting nuclear antigens in formalin-fixed, paraffin-embedded human neural tissues is difficult as the fixation process cross-links amino acids and nucleic acids potentially leading to the loss of antigen immunoreactivity (Evers et al., 1998; Ezaki, 2000). This decreases the ability to visualize proteins by antibody-antigen chemistry, and requires the use of antigen retrieval (AR) approaches to expose epitopes and enhance protein recognition (Ferrier et al., 1998; Shi et al., 2001a).

The purpose of the present study was to standardize an AR method for antibody-based detection of neuronal nuclear proteins in human formalin-fixed, paraffin-embedded tissue that may have been masked during tissue fixation and processing. We compared several AR methods using IHC and immunofluorescence (IF) detection methods with identification of the neuronal nuclear protein NeuN as the outcome measure; this is done with the view that these methods could be generalized for studying other nuclear proteins in human formalin-fixed, paraffin-embedded tissue. NeuN is a DNA-binding protein found exclusively in neurons with a molecular mass of 46–48 kDa (Lind et al., 2005; Mullen et al., 1992; Wolf et al., 1996). Although the function of NeuN is not known, it appears to be a nervous system-specific nuclear regulatory molecule (Soylemezoglu et al., 2003). This study illustrates some of the difficulties encountered when studying neuronal nuclear proteins by immunostaining, and provides solutions for exposing nuclear antigens to allow characterization of expression, distribution, and localization of endogenous nuclear proteins.

2. Materials and methods

2.1. Materials

We purchased NeuN antibody from Chemicon International (Temecula, USA). For the immunohistochemistry work, the Vectastain Elite ABC Kit and biotinylated goat-antimouse secondary antibody was purchased from Vector Laboratories (Burlington, ON, Canada), and the diaminobenzidine tetrahydrochloride (DAB) and trypsin was purchased from Sigma (Oakville, ON, Canada). The Contrast BLUE was purchased from KPL (Gaithersburg, USA). For the immunofluorescence work, the AlexaFluor 546-conjugated goat anti-mouse secondary antibody and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Molecular Probes (Eugene, OR, USA), and the Sudan black B and Permount were purchased from Fisher Scientific (Nepean, ON, Canada). For the antigen retrieval methods used, the Target Retrieval Solution S3307 (High pH, 10X concentrate), Target Retrieval Solution S1699 (Low pH, 10X concentrate), and proteinase K were obtained from DAKO (Mississauga, ON, Canada) and immuno-mount aqueous mounting medium was supplied by VWR (Mississauga, ON, Canada).

2.2. Tissue preparations

Fixed-frozen sections of Wistar rat spinal cord were kindly provided by Dr. L.C. Weaver (Robarts Research Institute). Briefly, rats were anesthetized and perfused transcardially with Dulbecco's modified Eagle medium containing 4% paraformaldehyde, as described previously (Krenz and Weaver, 1998). Spinal cords were removed and post-fixed in 4% paraformaldehyde overnight. The tissues were then taken through an ascending sucrose concentration gradient of 10, 20 and 30% overnight, followed by freezing the tissues at −80 °C. Tissues were freeze-thawed and mounted in O.C.T. compound on a standard cryostat. Thirty-micrometer transverse sections were cut from the thoracic spinal cord, then mounted onto slides and air-dried. Sections were processed according to the IHC protocol for fixed-frozen tissue described below.

Paraffin-embedded sections of mouse spinal cord were obtained from the laboratory of Dr. M.J. Strong (Robarts Research Institute). Mice were anaesthetized and perfused with heparinized saline, then fixation was achieved by intracardial injection of 4% paraformaldehyde with 0.1 M phosphate-buffered saline. Spinal cords were removed and placed in fixative overnight at 4 °C. Fixed tissues were processed by dehydration through a graded series of ethanol, cleared in xylene, then embedded in paraffin blocks using automated processing and embedding equipment. Sections were cut from blocks at 4 μm thickness and mounted onto slides.

Archival (between 1997 and 2002) formalin-fixed, paraffin-embedded necropsy human spinal cord tissue was obtained from the Department of Pathology at the London Health Sciences Center (University Hospital Campus), London, Ontario, Canada. Spinal cord tissue was processed routinely as follows. After removal, tissue samples were fixed by submersion in 10% neutral buffered formalin with the average period of fixation being about 24 h at room temperature. Fixed tissues were then processed by dehydration through a graded series of ethanol, cleared in xylene, then embedded in paraffin blocks using automatic processing and embedding equipment. Selection of paraffin blocks for study was based on examination of one hematoxylin and eosin-stained slide to establish the anatomical location in the tissue. Paraffin-embedded tissue sections were cut from blocks at 4 μm thickness and mounted onto slides.

2.3. Immunohistochemistry (IHC) and immunofluorescence (IF) methods

Human spinal cord sections were de-paraffinized in two xylene washes, then re-hydrated in decreasing concentrations of ethanol from 100 to 50% followed by washing with water. To inhibit endogenous peroxidase activity, tissue sections were treated with freshly prepared 3% H_2O_2 for 15 min and washed three times with distilled water. Sections were then processed according to various AR protocols, summarized in Table 1 and described in detail below. Following AR, tissue sections were incubated with the monoclonal primary antibody for NeuN at a dilution of 1:1000 overnight at 4 °C. Slides were washed with PBS, then incubated with the appropriate secondary antibody for either IF or IHC.

For IHC, the Vectastain Elite ABC Kit (mouse) was used for development of diaminobenzidine tetrahydrochloride (DAB) chromagen to allow detection of the primary antibody. Tissue sections were incubated with biotinylated goat-antimouse secondary antibody at a concentration of 1:200 for 1 h at room temperature, followed by signal amplification using the ABC reagents according to manufacturer's instructions. The slides were washed with PBS and tissue sections were stained with DAB at a concentration of 0.5 mg/ml in PBS containing 0.01% H_2O_2. Sections were counterstained with Contrast BLUE to highlight tissue morphology, then dehydrated with increasing concentrations of ethanol from 50 to 100% followed by two washes of xylene and cover-slipping using Permount histological mounting medium.

For IF, tissue sections were incubated with the secondary antibody AlexaFluor 546-conjugated goat anti-mouse IgG at a concentration of 1:400 for 1 h at room temperature. Sections were then washed with PBS and incubated with the nucleus-specific counterstain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to highlight all cell nuclei. Slides were cover-slipped using immuno-mount aqueous mounting medium and dried at 4 °C overnight in the dark. To quench the auto-fluorescence associated with the formalin-fixed, paraffin-embedded tissue the following reagents were tested: 0.3% Sudan black B (SBB) in 70% ethanol for 10 min at room temperature or 1 mg/ml $NaBH_4$ for 5 min. Slides were incubated with either of these reagents after treatment with the secondary antibody and prior to mounting of coverslips.

2.4. Antigen retrieval (AR) methods

Both enzymatic and heat-induced approaches were tested as AR methods, as described in Table 1. The heat-induced AR method was used in conjunction with buffers having different pH values. Three solutions were tested: 10 mM citrate buffer pH 6 prepared in our laboratory, commercially available target antigen retrieval solution high pH (referred to as alkaline pH AR buffer), and commercially available target antigen retrieval low pH (referred to as acidic pH AR buffer). Slides holding de-paraffinized tissue sections were heated in a bath containing one of the above mentioned buffers at either 95, 100 or 105 °C for either 15 or 20 min to test the optimal temperature and incubation time for AR. This was followed by a 1 h cool-down period before proceeding to the next step. The enzymatic AR methods used were a commercially available proteinase K solution (DAKO) for 5 min at room temperature or 0.25% trypsin incubation for 15 min at 37 °C.

2.5. Microscopy

Tissues stained with fluorescently labelled secondary antibody were visualized using either a Nikon Fluorescence E1000 microscope with a DXM1200 digital camera or a Zeiss LSM510-Meta scanning laser confocal microscope. When using the Nikon microscope, exposure times were reduced to yield a dark image for negative control sections, then images were captured using Act-1 software. Once set, the light intensity, exposure time, and the filter tubes used (FITC, Ex. 460–500 nm; Texas Red, Ex. 540–580 nm, and UV, Ex. 360–380 nm) were maintained at the same levels for all tissue sections imaged. Images from the confocal microscope were acquired digitally using a 63× oil immersion objective, using excitation (488 nm) and emission (515 nm) wavelengths and Zeiss LSM software. Tissues stained with the chromagen DAB were visualized with a Nikon Labophot-2 light

Table 1
Antigen retrieval methods used in conjunction with immunofluorescence and immunohistochemistry for detection of NeuN in nuclei of spinal cord neurons

Species	Tissue type	Antigen retrieval methods		NeuN staining	
				Immunofluorescence[a]	Immunohistochemistry
Human	Formalin-Fixed Paraffin-embedded	No AR	N/A	–	–
		Enzymatic AR	0.25% Trypsin	–	–
			Proteinase K	–	–
		Heat-induced AR[b]	10 mM Citrate buffer pH 6	–	–
			Alkaline citrate buffer	–	++
			Acidic citrate buffer	–	+
Rat	Fixed-frozen	No AR	N/A	++	N/A
	Fixed, paraffin-embedded	No AR	N/A	–	N/A
		Heat-induced AR[b]	Alkaline citrate buffer	–	N/A

'++' indicates strong staining; '+' indicates weak staining; '–' indicates absence of staining.
[a] All formalin-fixed, paraffin-embedded tissues were incubated with 0.3% Sudan black B to quench the intrinsic auto-fluorescent properties of the tissue.
[b] All tissue was incubated at 100 °C for 20 min.

microscope equipped with 10, 20 and 40× objectives, and images were captured using Northern Eclipse Version 6.0 software. All images were formatted using Adobe Photoshop Version 7.0 and CorelDraw Version 9.0.

3. Results

3.1. Staining of neuronal nuclear protein NeuN in fixed-frozen and paraffin-embedded sections of rodent spinal cord tissue

Several experimental variables can affect detection of nuclear proteins by immunostaining including: the method for fixation (i.e. perfusion versus immersion), the type of fixative, post-fixation tissue processing (i.e. paraffin-embedding), type of tissue sections (i.e. frozen versus paraffin-embedded), use of various AR approaches, and the detection method (i.e. fluorescence- versus chromagen-based). It is known that the immunoreactivity of antigens in tissues can be decreased by fixation, and that this is generally related inversely to the incubation period with fixative (Shi et al., 2001a). Thus the longer that a tissue undergoes fixation, the greater the risk that antigenic epitopes in proteins can become masked requiring the use of AR steps to try to reconstitute protein antigenicity. With perfusion-fixed frozen rodent tissues, the time between staining and fixation can be reduced, and thus antigenicity of proteins may remain more intact and treatment of tissue sections with AR methods is generally not required prior to immunostaining.

The objective of this part of the study was to assess if different post-fixation tissue processing approaches affected the ability to visualize the nuclear protein NeuN in rodent spinal cord. First, we stained fixed-frozen sections of spinal cord according to the IF protocol, and found that the NeuN antibody bound to its antigen and could be visualized readily in nuclei of neurons without incorporation of an AR step (Fig. 1A–F). Tissue sections were counterstained with DAPI to identify nuclei of all cells including neurons, thus allowing verification that the staining pattern of the NeuN antibody was neuron-specific and nuclear-localized. Insets included in Fig. 1A–C indicate that immunostaining of NeuN in neurons is localized to the nucleus. By comparison, when paraffin-embedded sections of perfusion-fixed rodent spinal cord were processed using the same IF protocol, no immunostaining for NeuN was observed (Fig. 1G–J). Incorporation of an AR step using Target Retrieval Solution (high pH, Dako) in this IF protocol did not result in recovery of immunostaining for NeuN (Fig. 1K–N).

3.2. Effects of AR on IF and IHC of formalin-fixed, paraffin-embedded human spinal cord tissue

As indicated previously, extended fixation of tissues in formalin can cause masking of antigenic sites on proteins. However, in the case of human necropsy tissue, tissues cannot be fixed by perfusion and more prolonged immersion fixation is required for adequate preservation of tissue morphology. This is typically followed by paraffin-embedding of tissue samples. Therefore, to visualize proteins in archival human tissues using histological methods, uncovering epitopes by AR can constitute a critical part of the staining process. We tested two types of AR techniques, heat-induced and enzymatic epitope recovery, on formalin-fixed, paraffin-embedded necropsy human spinal cord sections (Table 1). For heat-induced AR methods, the pH of the buffers can have a significant effect on the efficiency of antigen recovery (Evers and Uylings, 1994; Evers et al., 1998). Therefore, we tested 10 mM citrate buffer with moderate pH (pH 6) prepared in our laboratory, as well as two commercially available AR buffers with either acidic or alkaline pH. Three different temperatures (95, 100 or 105 °C) and two different incubation times (15 or 20 min) were tested in the AR protocols to determine which condition yielded staining patterns with the best signal-to-background ratio. In the case of the enzymatic AR methods, we tested 0.25% trypsin or a commercially available proteinase K solution. Furthermore, we tested the different AR methods for both IF and IHC detection.

From our evaluation of the different AR methods, it appears that the best staining results for the nuclear antigen NeuN were achieved by IHC with the commercially available alkaline pH AR buffer (Fig. 2 and Table 1). Of the conditions tested, we found that boiling tissue sections at 100 °C for 20 min gave the most intense IHC staining with the lowest background staining. It is interesting to note that when comparing the antigen–antibody complex detection procedures, different results were obtained for IF in comparison to IHC with DAB development for a given AR method. For example, as would be predicted from our studies performed on paraffin-embedded sections of rodent spinal cord (Fig. 1), we did not observe detectable staining for NeuN by IF for any of the AR conditions tested (Fig. 2B). Tissues processed for staining by IF were counterstained with DAPI to highlight cell nuclei, but no fluorescence co-localization with NeuN was observed (Fig. 2B and C). This is compared with excellent nuclear staining of NeuN in primary motor neurons of the human spinal cord with IHC when using the alkaline pH AR buffer in conjunction with heat-induction at 100 °C for 20 min (Fig. 2E). Faint staining was also observed in nuclei of some neurons in tissue sections processed with the commercially available acidic pH AR buffer using IHC detection (Fig. 2G), and no staining was found in the absence of AR treatment (Fig. 2I). Moreover, no staining was observed for either the IHC or the IF detection procedures used in conjunction with either of the enzymatic AR methods, indicating that the NeuN antigen was not effectively unmasked using these enzymatic approaches (data not shown).

3.3. Quenching of auto-fluorescence

Formalin fixation of tissues can also result in development of high background fluorescence referred to as primary

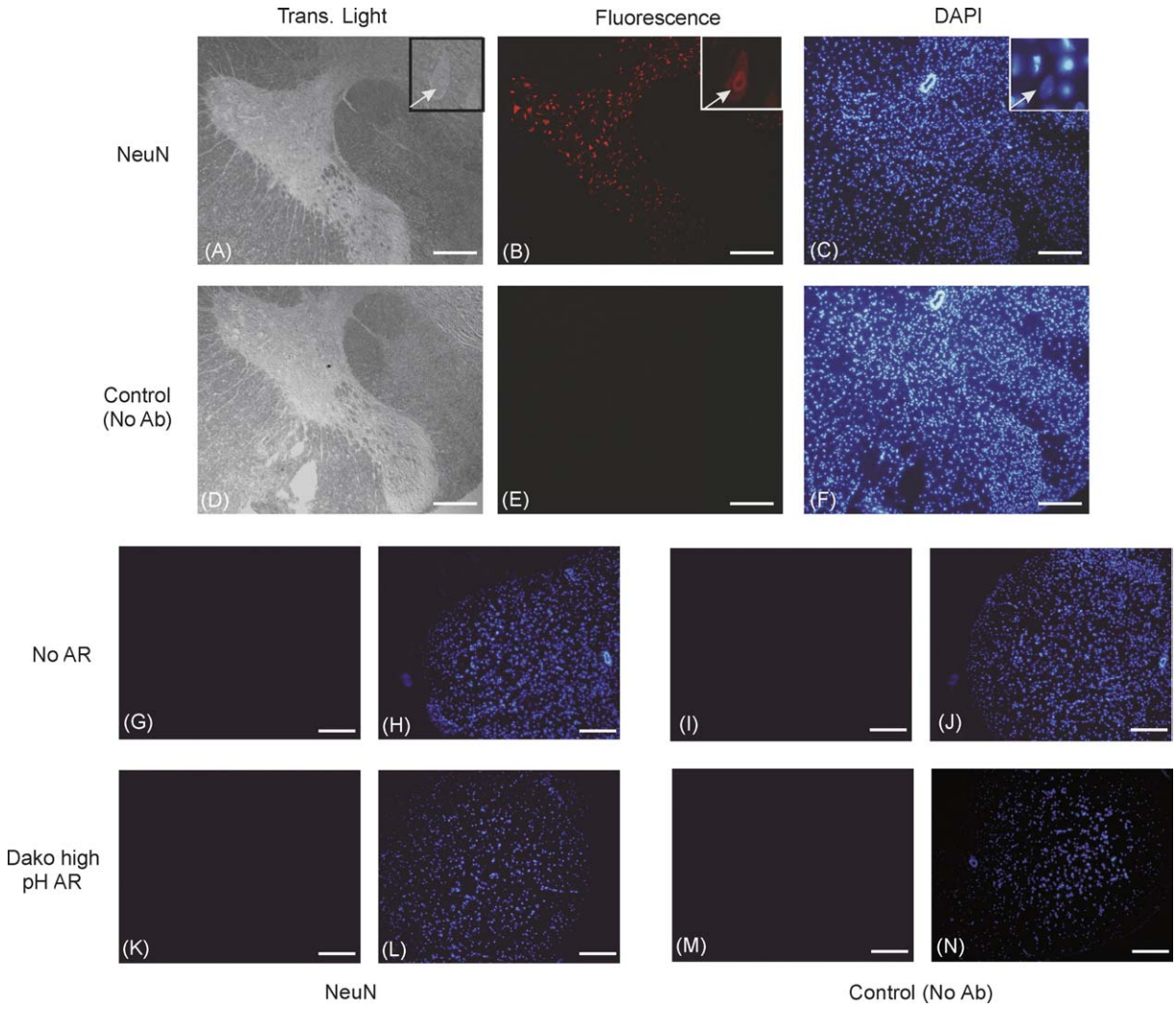

Fig. 1. Antigen retrieval was not required for staining of NeuN in fixed-frozen sections of rodent spinal cord. Panels A–F: frozen sections cut from perfusion-fixed rat spinal cord were incubated in the presence (A–C) or absence (D–F) of monoclonal anti-NeuN primary antibody and AlexaFluor 546-conjugated secondary antibody, and counterstained with DAPI to identify nuclei. Insets in panels A–C indicate that immunostaining for NeuN is found in the nuclei of neurons. Panels G–N: sections from paraffin-embedded perfusion-fixed mouse spinal cord were used to test the requirement for AR for detection of NeuN using IF detection. Panels G–J show results obtained without incorporation of an AR step, and panels K–N show results obtained when tissue sections were processed in parallel with AR using boiling in alkaline pH solution. Panels G and K show that IF staining of NeuN was not observed in paraffin-embedded tissues in either the presence or absence of AR. As a positive control in these experiments, paraffin-embedded sections of human spinal cord were processed in parallel with positive staining obtained with AR with boiling alkaline pH solution and development by IHC. Images were captured using a Nikon epifluorescence microscope, and scale bars represent 200 μm.

auto-fluorescence (Rost, 1980) (Fig. 3A and B). This can make the identification of structures positively stained by IF difficult because of a reduction in signal-to-noise ratio in formalin-fixed, paraffin-embedded tissue sections. Therefore, we tested two approaches for treating tissue sections to minimize this problem associated with IF staining protocols. In one method, spinal cord sections were treated with 0.3% SBB after the incubation with the secondary antibody to quench the intrinsic auto-fluorescence (Fig. 3E and F). SBB is highly lipid soluble, and thus dissolves readily in lipids that contribute to the tissue auto-fluorescence (Yao et al., 2003). In the second method, tissue sections were treated with 1 mg/ml $NaBH_4$ (Fig. 3C and D), which reduces aldehyde and ketone groups to their respective alcohols, thereby also serving to reduce tissue auto-fluorescence (Baschong et al., 2001). Of the two methods tested, SBB was more effective, and therefore was used on all formalin-fixed, paraffin-embedded tissue sections stained by the IF method.

3.4. Reducing Interference due to lipofuscin deposits

Lipofuscin is an endogenous cellular pigment that accumulates with increasing age in the cytoplasm of neurons (Yin, 1996). Because it has intrinsic auto-fluorescent properties (Fig. 4A and B), lipofuscin can compound problems with analysis of IF-stained human neural tissue. It can also cause problems with IHC because it distorts cellular structure. Endogenous tissue fluorescence related to lipofuscin is

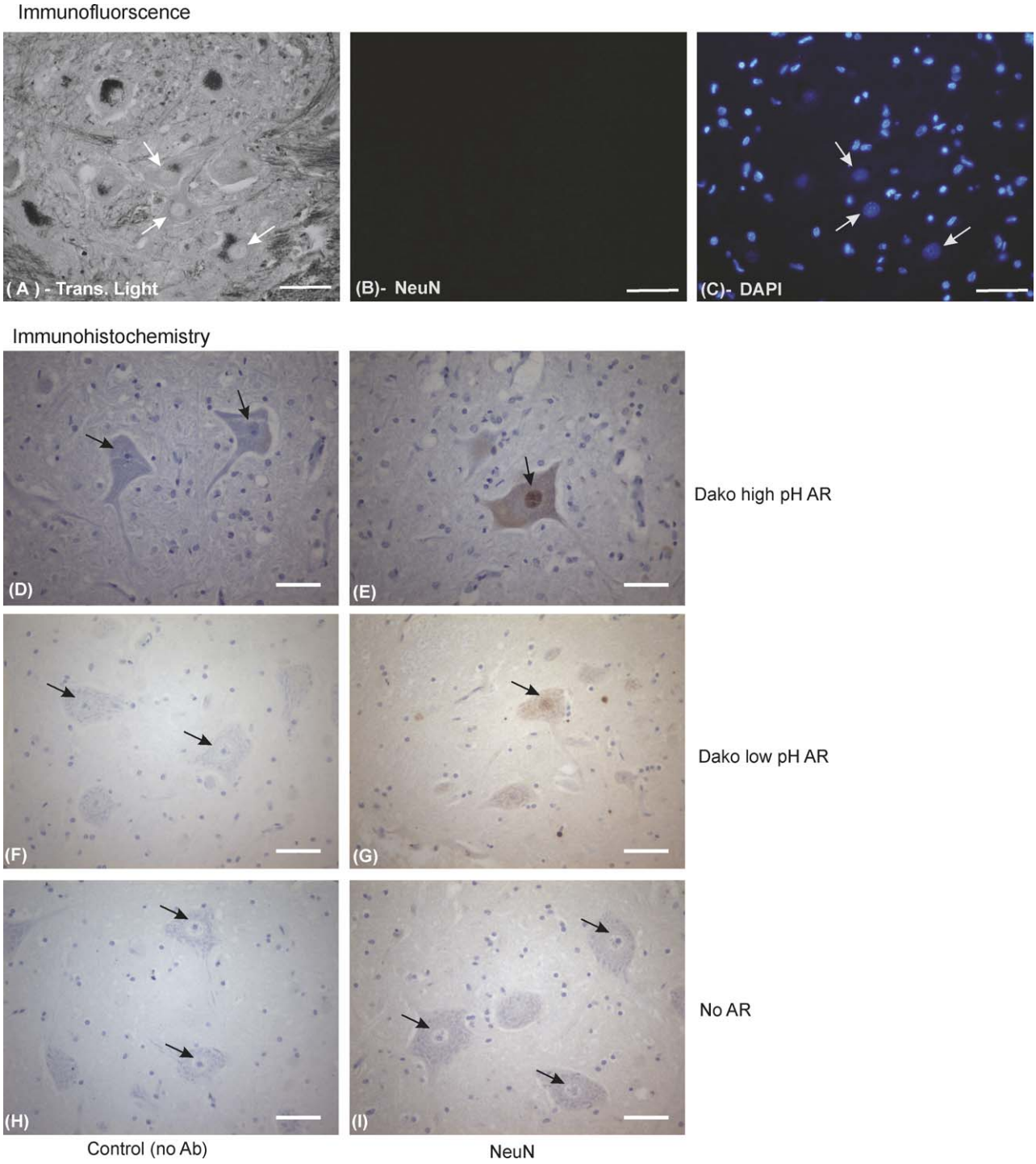

Fig. 2. Treatment of formalin-fixed paraffin-embedded human spinal cord sections with alkaline pH AR solution at elevated temperature reveals optimal staining of NeuN using IHC detection. Panels A–C: photomicrographs in panels B and C illustrate IF images, with panel A showing the associated tissue morphology with transmitted light. Panels D–I: these photomicrographs illustrate IHC images. Panels A–E, F and G, and H and I are from tissue sections treated with alkaline pH AR solution, acidic pH AR solution, or no AR treatment, respectively, as described in Methods section. Panel B shows a lack of IF-staining for NeuN IF, with panel C illustrating location of DAPI stained cellular nuclei. Panels D, F and H show control tissue sections processed for IHC in the absence of primary antibody, and stained with contrast blue. Panel E illustrates positive staining of NeuN in neuronal nuclei by IHC in sections treated with alkaline pH AR solution. Panels G and I show either weak or no staining for NeuN by IHC with either acidic AR solution or no AR treatment, respectively. The arrows depict nuclei of motor-neurons. Images in panels A–C were captured using the Nikon epifluorescence microscope, and images in panels D–I were captured by light microscopy. Scale bars represent 50 μm.

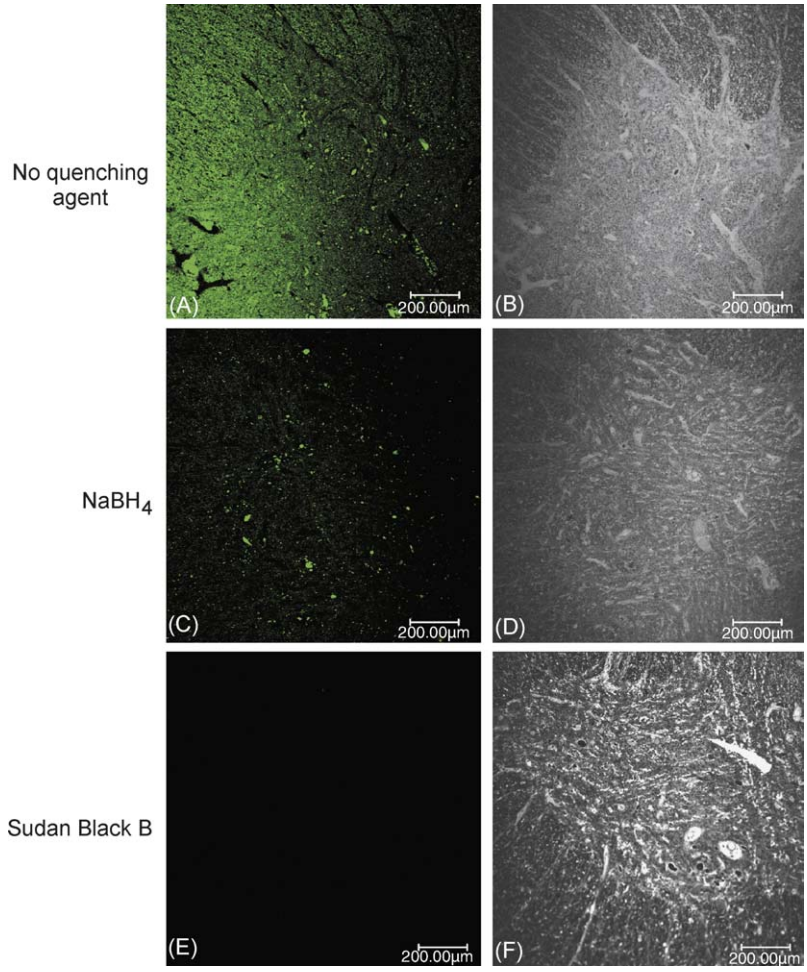

Fig. 3. The effects of quenching agents on the primary auto-fluorescence of formalin-fixed, paraffin-embedded human neural tissue. Panels A and B illustrate auto-fluorescence in tissue that was not treated with quenching agents. Auto-fluorescence appears to be reduced, but not eliminated, in tissues treated with $NaBH_4$ (Panels C and D). By comparison, tissue auto-fluorescence was eliminated by SBB-treatment (Panels E and F). Panels A, C and E are fluorescence images, and Panels B, D and F are images obtained with transmitted light. Scale bars represent 200 μm.

also classified as primary auto-fluorescence (Rost, 1980), and it has been reported that SBB can inhibit this by masking the cellular structures that contain lipofuscin (Baschong et al., 2001; Romijn et al., 1999). Therefore, we tested treatment of spinal cord sections with 0.3% SBB to determine if this could quench auto-fluorescence associated with lipofuscin, and thereby enhance our ability to detect antigen–antibody complexes in combination with the various AR methods on tissue sections stained by IF. We found that 0.3% SBB was effective in quenching the auto-fluorescent nature of lipofuscin (Fig. 4C and D).

4. Discussion

In this study, we tested methods that can be used to achieve immunostaining or to improve signal intensity for IHC or IF detection of neuronal nuclear proteins in formalin-fixed, paraffin-embedded sections of necropsy human spinal cord. The results of our study indicate that the AR method that is most effective in recovering antigenicity of formalin-fixed, paraffin-embedded modified nuclear protein NeuN is treatment with an alkaline pH AR buffer solution at high temperature. Moreover, we show that standard IHC with DAB chromagen development is more effective for visualization of nuclear antigens in archival neural tissue than is IF. Finally, we show that by incubating tissues with SBB, the intrinsic auto-fluorescence associated with formalin-fixed, paraffin-embedded human necropsy tissue or lipofuscin can be effectively quenched.

In general, frozen sections cut from perfusion-fixed tissues are more amenable for histological identification of low-abundance neuronal nuclear proteins than are formalin-fixed, paraffin-embedded archival tissues as AR is generally not required prior to the staining procedure. In the present study, we tested staining for NeuN on fixed-frozen sections of rodent spinal cord and found an abundant IF signal for this protein in neuronal nuclei without the use of an AR step, in agreement with published work by other groups (Mullen et al., 1992). Conversely, when performing the IF procedure

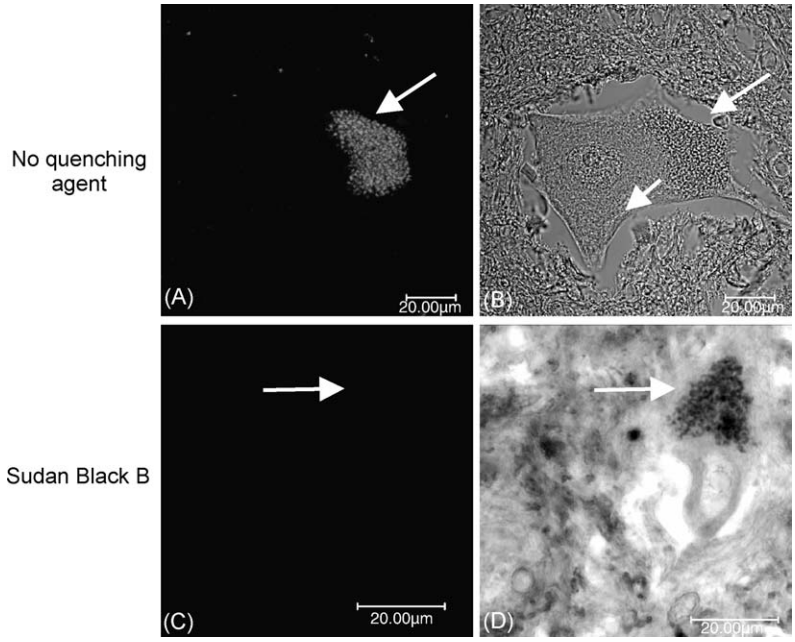

Fig. 4. Effects of Sudan black B on the auto-fluorescence associated with lipofuscin. Panels A and B show auto-fluorescence related to lipofuscin deposits in motor neurons in de-paraffinized, but unstained spinal cord sections. Panels C and D illustrate the masking effects of SBB on lipofuscin pigment, and the elimination of lipofuscin-associated auto-fluorescence. Panels A and C are fluorescence images, and panels B and D are transmitted light images. The arrows identify lipofuscin deposits. Images were captured by confocal microscopy. Scale bars represent 20 μm.

on perfusion-fixed, paraffin-embedded rodent spinal cord tissue, no IF signal was observed, indicating that AR methods were necessary to expose the nuclear antigen.

Although frozen sections of formalin-fixed human neural tissues can be obtained and are suitable for some purposes, it is more common that human necropsy samples are formalin-fixed and paraffin-embedded, particularly when retrospective studies are being conducted. These tissues have the advantage of ease of long-term storage and better preservation of tissue morphology related to the longer fixation and paraffin-embedding process (Beckstead, 1995; Onodera et al., 1992; Shi et al., 2001a). Unfortunately, these benefits also coincide with the major disadvantage that the longed tissue fixation and embedding process can mask protein antigenic sites and decrease or even eliminate recognition by antibodies (Shi et al., 2001a). Therefore, identifying low-abundance nuclear proteins in human formalin-fixed, paraffin-embedded tissue is challenging and generally requires the use of appropriate AR methods. We tested AR methods, listed in Table 1, that fall broadly into two classes: heat-induced epitope recovery or enzymatic epitope recovery (Shi et al., 1991; Shi et al., 2001b).

Heat-induced AR is based on the principle that the combination of heat and pH can restore antigenicity to protein epitopes (Shi et al., 2001a). In general, using weak buffers, rather than water, at elevated temperatures is most effective for this purpose (Shi et al., 1997). The optimal temperature must be determined empirically, but typically shorter incubation intervals are required as the temperature is increased (Shi et al., 2001a). Moreover, the pH of the solutions used during AR also has a dramatic effect with the most commonly tested buffers having acidic (2–5), neutral (6–8) or alkaline (9–11) pH (Evers and Uylings, 1994; Shi et al., 1995; Shi et al., 2001a). Enzymatic AR methods have also been used to recover immunoreactivity of proteins, with de-paraffinized tissue sections incubated with a protease such as trypsin, pepsin or proteinase K (Miller et al., 2000). It is likely that these proteolytic enzymes break formaldehyde-induced methylene cross-links in the antigenic molecules thus restoring immunoreactivity (Leong et al., 2002), but prolonged exposure to enzymes can also be detrimental (Leong et al., 1988). Heat-induced and enzymatic AR have also been used in combination to achieve good staining results (Ezaki, 2000; Leong et al., 2002a).

These AR approaches have been used successfully to recover antigenicity to multiple proteins in several tissue or cell types, but in the current study we show that this methodology can be applied for immunostaining of an antigen in nuclei of human neural tissue. Our current findings with the NeuN protein, are supported by studies on other nuclear antigens such as the proliferating cell nuclear antigen (PCNA) (Ezaki, 2000), and the androgen receptor (Shi et al., 1993). Here also, heat-induced AR was found to be more effective than enzymatic AR using trypsin or other proteases to enhance antigenicity of a nuclear protein (Ezaki, 2000; Shi et al., 1993). Our studies focus on exposing nuclear antigens in human neural tissue, whereas other studies have used human prostate and appendix tissues or rat intestine, liver and thymus to assess recovery of antigenicity of nuclear proteins for immunostaining.

Interestingly, we also find a difference between IF and IHC staining when tested with a given AR method. Positive staining of NeuN was observed with alkaline pH AR buffer and IHC, but not with the IF protocol. Positive neuron-specific nuclear NeuN IF staining was obtained on the fixed-frozen rodent tissue, but not in sections from paraffin-embedded rodent spinal cord. Therefore, it is unlikely that the absence of staining of NeuN in the formalin-fixed, paraffin-embedded human tissue is related to the general IF procedure. It is also unlikely that the absence of staining using the IF method is a result of inadequate AR in the tissue sections as positive staining was obtained with IHC. Several reasons may account for the differences observed with staining between the two detection methods. IF staining on formalin-fixed, paraffin-embedded human neural tissue is complicated by endogenous auto-fluorescence of the tissue caused by the fixation process (Baschong et al., 2001). Formalin-fixation causes cross-linking to occur between proteins leading to oxidation of aldehyde groups, which in turn fluoresce when the tissue is excited by light at certain wavelengths (Fig. 3). This results in a decrease in the signal-to-noise ratio and difficulty in interpreting IF results. Furthermore, an added complexity of working with neural tissue is human neurons contain specific cellular constituents, such as lipofuscin, that also exhibit auto-fluorescent properties (Baschong et al., 2001; Neumann and Gabel, 2002; Romijn et al., 1999). The lipofuscin pigment that accumulates in non-dividing cells such as neurons consists of lipids, proteins, and degraded lysosome organelles (Neumann and Gabel, 2002; Yin, 1996). These lipofuscin deposits complicate signal-to-noise IF signals making it difficult to observe positive staining in tissue.

Methods have been described that mask or quench auto-fluorescence associated with formalin-fixation and lipofuscin in neural tissue (Baschong et al., 2001). We incubated spinal cord sections with 0.3% SBB and successfully quenched auto-fluorescence and masked cellular structures that contain lipofuscin (Baschong et al., 2001; Romijn et al., 1999). However, treatment of tissue sections with chemicals to block auto-fluorescence can also reduce the intensity of IF labelling (Neumann and Gabel, 2002). Therefore, one explanation for the absence of IF signal in the formalin-fixed, paraffin-embedded spinal cord sections in the present study could be related to excessive quenching by SBB. Unfortunately, without this experimental step, the auto-fluorescence was too strong to detect positive staining for NeuN in the tissue sections. It is important to note that the fixed-frozen, but not paraffin-embedded, rodent spinal cord tissue sections stained positively for NeuN by IF, but this tissue did not exhibit auto-fluorescence at levels seen with the human tissues, and therefore SBB treatment was not required.

The IHC detection method used in the present study involved an additional signal amplification step that is not present in the IF approach. The amplification step uses ABC technology (Vector Laboratories), which amplifies the DAB signal thereby facilitating detection of proteins that are expressed at low to moderate levels. Since we are studying expression of low-abundance endogenous nuclear proteins, it is likely that an amplification step is required for reliable detection. In the present studies, we attempted to enhance the IF staining for NeuN using the tyramide signal amplification (TSA) technology (Molecular Probes). TSA is an enzyme-mediated detection method that uses the catalytic activity of horseradish peroxidase (HRP) to generate high-density labeling of a target protein in situ (Speel et al., 1999). However, a generalized increase in auto-fluorescence was seen throughout the tissue when TSA was used resulting in a decrease in signal-to-background, and an inability to distinguish positive staining from auto-fluorescence (data not shown).

5. Conclusion

In summary, we investigated the effectiveness of different AR methods on recovery of antigenicity of the nuclear protein NeuN in neurons of formalin-fixed, paraffin-embedded necropsy human spinal cord tissue. Our data indicate that AR using an alkaline pH buffer at elevated temperature is required for immunostaining of NeuN in these human archival tissues by IHC.

Acknowledgements

These studies were supported by an operating grant from the Canadian Institutes for Health Research (CIHR) to RJR, and Studentships from Alzheimer Society of Canada, Ontario Graduate Studentship in Science and Technology, and a CIHR Canada Graduate Scholarship Doctoral award to SKG. The authors thank Dr. K. Leco (University of Western Ontario) for use of the Nikon epifluorescence microscope, Dr. M.J. Strong (Robarts Research Institute) for providing the perfused-fixed, paraffin-embedded mouse spinal cord tissue, use of the Nikon light microscope and helpful discussions, and Dr. L.C. Weaver (Robarts Research Institute) for providing perfused-fixed rat spinal cord tissue.

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