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ALL. 2

Selezione pubblica, per titoli ed esami, per l'assunzione di n. 1 unità di personale di Categoria **D1** - area tecnica, tecnico-scientifica ed elaborazione dati - con contratto di lavoro subordinato a tempo indeterminato, presso il Dipartimento di Medicina Sperimentale e Clinica - (Selezione indetta con D.D.G. n. 262 del 14.7.2020)

Prova ORALE del 5/03/2021

TRACCIA 1

Quesito

Fasi di preparazione di un omogenato di corteccia cerebrale di topo per lo studio al western blotting di una proteina a prevalente espressione citoplasmica e fasi di preparazione del tessuto cerebrale per l'allestimento di sezioni immunoreagite in fluorescenza doppio marcate per la localizzazione al microscopio confocale della stessa proteina nella corteccia cerebrale intatta.

Lingua inglese

Lettura e traduzione del testo evidenziato in giallo (*estratto da Park, K. W., Lee, H. G., Jin, B. K., & Lee, Y. B. (2007). Interleukin-10 endogenously expressed in microglia prevents lipopolysaccharide-induced neurodegeneration in the rat cerebral cortex in vivo. Experimental & molecular medicine, 39(6), 812-819).*

or how microglial activation is suppressed upon inflammation of the brain parenchyma.

In the present study, we therefore examined whether LPS injection into rat cerebral cortex induces endogenous expression of IL-10 in microglia *in vivo*. We also sought to determine whether IL-10 contributes to the downregulation of inflammation, and whether IL-10 might thus increase neuronal survival.

Materials and Methods

Animal treatments

Female Sprague-Dawley rats (230-280 g) were anesthetized with chloral hydrate (400 mg/kg I.p.), positioned in a stereotaxic apparatus, and LPS (Sigma, St. Louis, MO) was delivered unilaterally into the right cerebral cortex (anteroposterior 1.4 mm, mediolateral 2.0 mm, dorsoventral 2.0 mm from bregma). 5 μg of LPS was injected at a rate of 0.2 μl/min with a 30-gauge Hamilton syringe attached to an automated microinjector (Buwon, Seoul, Korea). For neutralization of IL-10, some animals received co-injection of LPS and anti-murine IL-10 neutralizing antibody or nonspecific murine IgG as a control (1 μg; R&D Systems, Minneapolis, MN).

Immunohistochemistry

Brain sections were incubated with the appropriate primary antibodies against mouse OX-42 (1:400; Serotec, Oxford, UK) for microglia, against mouse glial fibrillary acidic protein (GFAP, 1:500; Sigma) for astrocytes, and against NeuN (1:500; Chemicon International, Temecula, CA) for neurons. The following day, sections were incubated with appropriate biotinylated secondary antibody followed by avidin-biotin complex (Elite Kit from Vector Laboratories, Burlingame, CA). The bound antiserum was visualized by incubating with 0.05% diaminobenzidine-HCl (DAB) and 0.003% hydrogen peroxide in 0.1 M phosphate buffer (PB).

Double-immunofluorescence staining

For immunofluorescence staining, the brain sections were incubated with a combination of a mouse OX-42 (1:400) and goat anti-IL-10 (1:150); mouse anti-GFAP (1:400; Sigma) and goat anti-IL-10; mouse anti-NeuN (1:400) and goat anti-IL-10; mouse OX-42 and goat anti-IL-1β (1:200; R&D Systems); mouse OX-42 and rabbit anti-iNOS (1:200; Chemicon); mouse OX-42 and goat anti-TNF-α (1:50; R&D Systems). After washing, the sections were subsequently incubated with FITC-labeled anti-mouse IgG (1:200; Kirkegaard & Perry Laboratories, Gaithersburg, MD) and Texas red-labeled anti-rabbit IgG (1:200; Vector) or Texas Red-labeled anti-goat IgG (1:200; Vector). Tissues were mounted with Vectashield mounting medium (Vector) and viewed using an Olympus IX71 confocal laser scanning microscope (Olympus; Tokyo, Japan).

RT-PCR

Brains from the ipsilateral cortex after injection were used for RNA isolation using Trizol (Life Technology, MD). The reverse transcribed cDNA (2 μg of RNA) was subjected to PCR amplification with following primer sets. For rat IL-10, 5'-TGCCTTCAGTCAAGTGAAGAC-3' (sense) and 5'-AAACTCATTCATGGCCTTGTA-3' (antisense) and for rat IL-1β, 5'-CTCCATGAGCTTTGTACAAGG-3' (sense) and 5'-TGCTGATGTACCAGTTGGGG-3' (antisense) and for rat TNF-α, 5'-GTAGCCCACGTCGTAGCAAA-3' (sense) and 5'-CCCTTCTCCAGCTGGGAGAC-3' (antisense) and for rat iNOS, 5'-CACCTACTTCCTGGACATCACTAC- 3' (sense) and 5'-GTACTCTGAGGGCTGACACAAG-3' (antisense). The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 57°C for 30 s (TNF-α, IL-10, IL-1β) or 60°C for 30 s (iNOS), and extension at 72°C for 90 s for 25 cycles. GAPDH was also amplified as an internal PCR control using the following primers, 5'-TCCATGACAACTTTGGCATCGTGG-3' (sense) and 5'-GTTGCTGTTGAAGTCACAGGAGAC-3' (antisense). PCR products were separated by electrophoresis, stained with ethidium bromide, and then detected using UV light.

In situ detection of O_2^- and O_2^--derived oxidants

Hydroethidine histochemistry was performed for *in situ* visualization of O_2^- and O_2^--derived oxidants (Wu *et al.*, 2003). After injection, hydroethidine (1 mg/ml; Molecular Probes, Eugene, OR) was administered intraperitoneally. The brain sections with oxidized hydroethidine product, ethidium, were examined by confocal microscopy (Olympus).

Western blot analysis

For subcellular fractionation, protein extracts of both the cytosolic and membrane factions were prepared. Protein (30 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with following primary anti-

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Lingua inglese

Lettura e traduzione del testo evidenziato in giallo (estratto da *Jonkman, J., Brown, C. M., Wright, G. D., Anderson, K. I., & North, A. J. (2020). Tutorial: guidance for quantitative confocal microscopy. Nature protocols, 15(5), 1585-1611).*

microscopy (EM), offer superior preservation of some samples, including worms and yeast[6]. In chemical fixation, the trick is to address what is known as the immunocytochemical compromise—sufficient cross-linking is necessary to preserve morphological details, but too many cross-links can reduce antigenicity and prevent antibody penetration. Hence, fixation must be optimized for each target structure and antibody. Very stable tissue structures (e.g., cell-cell junctions) are easily retained in place, such that gentle methanol fixation may suffice for the purposes of identification[7], while other tissue components, such as soluble proteins, require more stringent fixatives such as aldehydes. The choice of imaging approach will also dictate the method to be used, since EM and even super-resolution microscopy approaches will reveal more subtle changes to the tissue architecture than lower-resolution approaches. Thus, while formaldehyde (FA) fixation is typically used in most fluorescence microscopy protocols, the bivalent cross-linker glutaraldehyde is commonly added in EM protocols. Importantly, certain cellular structures, such as microtubules, are also best preserved for fluorescence imaging using low concentration glutaraldehyde fixatives[4]. Be aware of the different sources of FA; formalin solution, commonly used by pathologists, also contains methanol; hence, FA prepared in pure solution from paraformaldehyde powder is generally superior to methanol formalin solution for immunocytochemistry. The duration and temperature of fixation must also be optimized. For antibodies or structures that do not withstand aldehyde fixation well, it can be helpful to try fixation using ice-cold methanol, acetone or ethanol. Conversely, the fixation of cytoskeletal structures can often benefit from warming the fixative (e.g., 37 °C), particularly for mammalian cells, to prevent a cold shock to the cells and disassembly of the cytoskeletal structures. Note that polyclonal antibody staining is generally more resistant to chemical fixation than staining with monoclonal antibodies, since they detect multiple epitopes[8]. Researchers should refer to literature on their specific tissues, structures and molecular targets of interest to determine the optimal fixation protocol for their experiments, and should also be aware of new, improved tissue preservation methods that are constantly being developed[9,10] and should be tested.

Permeabilization

Antibodies and fluorophores are generally unable to penetrate the plasma membrane and reach the cytoplasm, unless detergents are used to make holes in the membranes. The permeabilization regime and detergent choice must be optimized. Some structures are better revealed by a brief permeabilization step prior to or during fixation, but it is important to realize that soluble proteins will be removed during this process. If soluble proteins are the target of the staining, this is obviously problematic, but if other structures (e.g., focal adhesions) are the target, permeabilization during fixation can remove cytosolic proteins, thereby reducing background signal and improving image contrast. In order to retain soluble proteins, however, most protocols either fix first, or fix and permeabilize simultaneously. We recommend trying combinations of different steps to determine the best results for the target of interest. Insufficient permeabilization of tissue sections can lead to poor antibody penetration only into the top and/or bottom of the tissue, while too much permeabilization can lead to loss or artifactual redistribution of the target[11]. Triton-X is a harsher detergent than saponin, which interacts selectively with membrane cholesterol, producing small holes in the plasma membrane without affecting cholesterol-poor membranes of the mitochondria and the nuclear envelope[12]. However, saponin's effects are reversible, so it must be included in all solutions during the entire labeling procedure. Finally, when labeling surface proteins, it should be noted that the small amount of methanol in formalin can cause some cell permeabilization and allow labeling also of internal structures.

Labeling

The next step is to select an appropriate labeling method to specifically target and visualize the structure of interest. This could involve antibody staining or a direct labeling approach (the latter may negate the need for permeabilization). Genetic approaches, fluorescent proteins (FPs) or tags such as SNAP-tags and HALOtags[13], have been increasingly used over the past decades, but antibody approaches are still prevalent and useful for verifying the localization of genetically tagged proteins to the correct cellular structures. The use of nanobodies to couple more photostable organic dyes to genetically expressed FPs is particularly useful for super-resolution microscopy techniques[14] but can also be applied for confocal microscopy. Companies also sell reagents for tagging specific cellular structures, such as DNA dyes for the nucleus or fluorescent markers for specific organelles (membranes, lysosomes, mitochondria, etc.)[15,16].

There is no such thing as a 'standard' immunolabeling protocol for cells or tissues: entire books have been written on this topic alone[6]. Blocking steps (e.g., with BSA or serum) should be introduced when required (inevitably for tissues, often for cell monolayers), to minimize non-specific binding of primary or secondary antibodies that can lead to background fluorescence, or to block endogenous enzyme activity when using enzymatic labeling approaches. Appropriate antibody concentrations must be established, as well as the optimal duration and temperature of labeling. Although counter-intuitive, reducing antibody concentrations often leads to better staining as specific binding is retained at the expense of non-specific binding. The optimal duration can vary widely from 20–30 min for cultured cells to hours or even days for tissue sections or cleared organs. If labeling at room temperature results in a high level of background signal, incubation of the sample with primary antibody overnight at 4 °C may increase specificity. Incubation times with secondary antibodies (typically 30 min to 1 h) can be reduced to lower nonspecific background. Sufficient washing steps are also critical, with many, shorter washes being superior to a couple of long ones. Also note that total labeling and washing times for weakly fixed tissue or cells should be minimized to prevent loss of cellular components from the sample. Finally, a variety of controls are essential for both primary and secondary antibodies, including minus primary antibody and isotype controls[8]. Positive controls, such as overexpression of the protein of interest or expression in a cellular system that does not express the protein