Detecting Reactive Oxygen Species by Immunohistochemistry

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Summary

In cultured cells, an increase in cellular levels of reactive oxygen species (ROS) can be detected using multiple techniques including colorimetric assays, immunoblotting, and immunofluorescence. These methods can also be applied for ROS measurement in tissue samples, but often require tissue homogenization, and therefore do not distinguish between the different cell types within a tissue. Here, we describe a detailed protocol for determination of altered oxidative stress levels in different cell types in tissues, by detecting ROS-caused alteration of macromolecules using immunohistochemistry (IHC). This method is demonstrated by using 4HNE as a marker for lipid peroxidation in mouse pancreas tissue that contains precancerous lesions high in cellular oxidative stress.

Keywords

reactive oxygen species; immunohistochemistry; lipid peroxidation; 4HNE

1. Introduction

Aberrant, net accumulation of reactive oxygen species (ROS) in cells and tissues has been implicated in numerous diseases such as diabetes, neurodegenerative disorders and cancer, but also shortening of lifespan and organismal aging. Reactive oxygen species (ROS) are highly reactive molecules containing oxygen with unpaired electrons. Generation of ROS can be induced by environmental and other extracellular sources or internally in cellular organelles during biological processes. Inside a cell, ROS are generated as byproducts in many organelles including mitochondria, endoplasmic reticulum and peroxisomes. These highly reactive molecules not only attack DNA to cause DNA damage and adduct formation (i.e. DNA double strand breaks, 8-hydroxy-2-deoxyguanosine/8-oxo-dG), but also lead to protein oxidation (i.e. nitro-tyrosine) and lipid peroxidation (i.e. 4 hydroxy-2-noneal/4HNE, malondialdehyde). Elimination of excess cellular ROS is mediated by scavenging systems including superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxins (1). Since long-term imbalance between cellular ROS production and elimination has been implicated in organismal aging and onset and progression of numerous disorders including neurodegenerative diseases and cancer, it is important to be able to evaluate cellular ROS levels in clinical patient tissue samples or in animal models recapitulating disease (1, 2). In this chapter, we provide an immunohistochemistry protocol to assess cellular oxidative

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stress levels, using mouse pancreatic precancerous lesions and the lipid peroxidation product 4HNE as an indicator of ROS. With minor modifications (i.e. first antibody, and adjustment of dilution) this protocol can be applied to also detect DNA adducts, protein oxidation and other lipid peroxidation products (Table 1) by immunohistochemistry in any tissues of interest.

2. Materials

2.1. Buffers

1. Sodium citrate buffer pH 6.0: 10 mM sodium citrate, 0.05 % Tween 20, in distilled H_2O. Adjust pH to 6.0.

2. Phosphate-buffered saline (PBS): Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml of distilled H_2O; then adjust pH to 7.4; and then adjust volume to 1 L with additional distilled H_2O.

3. TBST buffer pH 7.6: 50 mM Tris, 150 mM NaCl, 0.05 % Tween 20, in distilled H_2O. Adjust pH to 7.6.

4. Blocking buffer: 5 % goat serum in $1\times$ TBST buffer.

5. 95% ethanol solution: 95 mL 100% ethanol plus 5 mL distilled H_2O.

6. 80% ethanol solution: 80 mL 100% ethanol plus 20 mL distilled H_2O.

7. 3% H_2O_2 solution: 5 mL 30% H_2O_2 (commercially-available) plus 45 mL distilled H_2O.

2.2. Immunohistochemistry

1. Rabbit anti-4HNE sera from Alpha Diagnostic International Inc. (San Antonio, TX, USA); or other antibodies directed against antigens that can serve as readout for increased oxidative stress (Table 1).

2. HRP-conjugated goat-anti-rabbit antibody (or other HRP-conjugated secondary antibody directed against the species in which the primary antibody was raised).

3. 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (available from multiple vendors).

2.3. Other Materials

1. Tissue slides with tissue of interest.

2. Pressure cooker or steamer.

3. Staining jar or holder (use polyethylene instead of glass).

4. Pap pen (optional).

5. Sharp-end forcep tweezers.

6. Standard IHC mounting medium (available from multiple vendors).

7. Coverslips.

3. Methods

Unless otherwise specified, carry out all procedures at room temperature (20 °C).

3.1. Deparaffinization of Tissue Slides

1. Incubate tissue slides in xylene for 5 min.

2. Repeat step 1 for another two times (*see* Note 1).

3.2. Rehydration of Tissue Slides

1. Perform 3 washes with 100% ethanol (*see* Note 2), 3 min for each wash.

2. Perform 2 washes with 95% ethanol, 3 min for each wash.

3. Perform 2 washes with 80% ethanol, 3 min for each wash.

4. Rinse tissue slides in distilled water for 5 min, twice.

3.3. Antigen Retrieval

1. Heat tissue slides (*see* Notes 3 and [4]) in sodium citrate buffer pH 6.0 at 95 – 100 °C for 20 min.

2. Remove the heat and let tissue slides cool in sodium citrate buffer on the bench until the temperature reaches room temperature (*see* Note 5).

3. Wash tissue slides with phosphate buffered saline (PBS) for 5 min, three times (*see* Note 6).

3.4. Immunohistochemical Staining of Tissue Slides

1. Incubate tissue slides with 3% hydrogen peroxide for 10 min.

2. Wash tissue slides with PBS for 5 min; repeat this step three times.

3. Place tissue slides in blocking buffer for 1 h at room temperature.

4. Prepare 4HNE antibody solution by adding rabbit anti-4HNE antibody to blocking buffer at a dilution of 1:600 (*see* Note 7).

5. Remove tissue slides from blocking buffer.

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[1]Xylene is flammable and a health hazard. This step should be carried out in a chemical fume hood with outdoor exhaust ventilation.
[2]Ethanol is flammable and a hazardous material. This step should be carried out in a chemical fume hood with outdoor exhaust ventilation.
[3]A pressure cooker or steamer is ideal for this key step because it can maintain a fairly constant temperature nearing boiling point.
[4]Do not use a glass staining jar or holder in this step because they will crack at high temperature. A polyethylene staining jar which can endure high temperature is best suitable for this process. In addition, using a polyethylene staining jar inside the streamer reduces the amount of sodium citrate buffer to be used.
[5]Make sure that the surface of the tissue sample area is always moist and kept in buffer or solution.
[6]Immediately proceed to the immunostaining procedure is highly recommended after antigen retrieval.
[7]This dilution (1:600) for the 4HNE antibody is optimized for mouse pancreas tissues. For other tissue types, either mouse or human, optimal dilution can vary and needs to be determined.

6. Apply 4HNE antibody solution to tissue sample area (*see* Note 8) and incubate overnight at 4 °C (*see* Note 9).

7. Remove 4HNE antibody solution from tissue slides.

8. Wash tissue slides with TBST buffer for 5 min; repeat this step 3 times.

9. Prepare secondary antibody solution by adding HRP-conjugated goat-anti-rabbit antibody in blocking buffer at a dilution recommended by the manufacturer.

10. Remove tissue slides from TBST buffer.

11. Apply HRP-conjugated goat-anti-rabbit secondary antibody solution to the tissue slide (*see* Note 10) and incubate for 30 min at room temperature (20 °C).

12. Remove secondary antibody solution from tissue slides.

13. Wash tissue slides with TBST buffer for 5 min; repeat this step 3 times.

14. Prepare DAB substrate solution according to the manufacturer's instructions.

15. Apply DAB substrate solution to tissue slides. Ensure that the substrate solution completely covers the tissue sample area.

16. Watch closely as tissue sample color turns into brown (*see* Note 11). When the signal intensity reaches the ideal condition (not too light, not too dark; Fig. 1), stop the reaction by immersing slides in distilled water.

17. Wash tissue slides with distilled water for 5 min, twice.

3.5. Dehydration and Mounting of Tissue Slides

1. Incubate tissue slides with 80% ethanol for 10 seconds, twice (*see* Note 12).

2. Incubate tissue slides with 95% ethanol for 10 seconds, twice.

3. Incubate tissue slides with 100% ethanol for 10 seconds, twice.

4. Incubate tissue slides with xylene for 10 seconds, twice.

5. Apply mounting media to the tissue slides and apply coverslips (*see* Note 13).

Acknowledgement

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[8]If you want to decrease the amount of 4HNE antibody solution that will be used in the next step, create a water-proof barrier by circling the tissue sample area on the glass slide using a pap pen.

[9]To avoid the 4HNE antibody solution from drying out during overnight incubation, the tissues slide can be placed on top of a wet paper towel in a sealable plastic container. This preserves the moisture inside the container.

[10]Ensure that the goat-anti-rabbit antibody solution completely covers the tissue sample area.

[11]The time period of incubation to develop signals varies and depends on the (DAB) peroxidase substrate kit used (see manufacturer's instructions). To test the incubation time for optimal signal intensity, it is recommended using tissue samples that have high levels of cellular ROS and control tissue slides (*see* Fig. 1). At ideal development time, a clear difference of 4HNE signal intensity between positive and negative tissue samples should be observed.

[12]Steps 1 – 4 should be performed in a chemical fume hood with outdoor exhaust ventilation.

[13]To avoid generating air bubbles between tissue sample area and coverslip, drop mounting media directly on top of the tissue area. Then use sharp-end forcep tweezers to hold the coverslip at one end and let the other end of the coverslip stand on the tissue slide. Slowly close the gap between tissue slide and coverslip by putting down the coverslip entirely.

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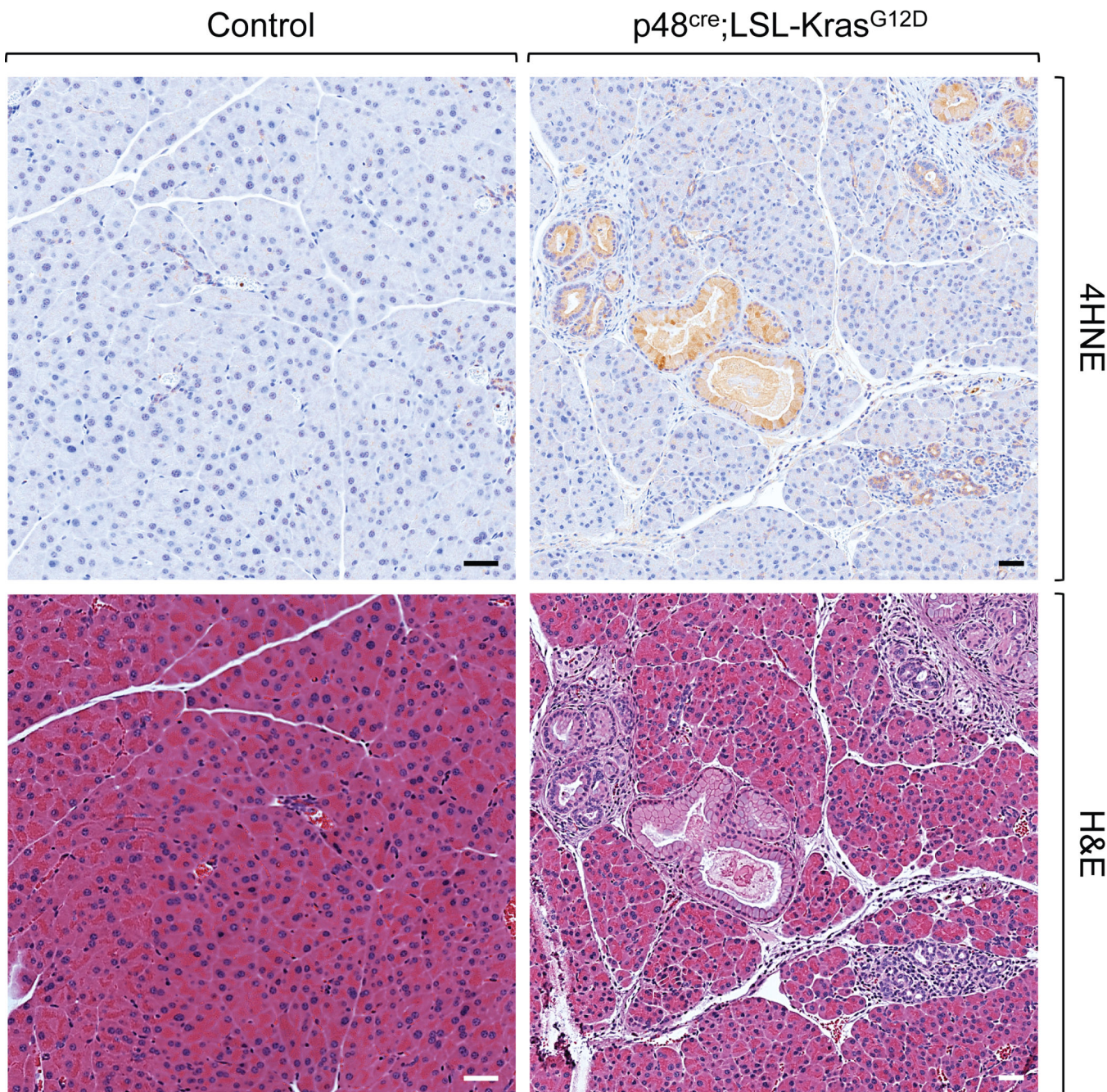
Fig. 1.

Detection of elevated ROS levels in pre-cancerous lesions. Staining of pancreatic tissues from a control mouse and a p48^{cre};LSL-Kras^{G12D} mouse, in which Kras with a proto-oncogenic mutation (Kras^{G12D}) was expressed under a pancreatic cell-specific transcription factor (p48). Expression of Kras^{G12D} leads to pre-cancerous pancreatic lesions that show high levels of oxidative stress. Tissues were fixed in formalin and then subjected to 4HNE immunohistochemistry (*top panel*) as described in this chapter. Additional hematoxylin & eosin (H&E) staining was performed to show pancreas morphology (*bottom panel*). The data

shows that our protocol is effective to detect oxidative stress-mediated lipid oxidation in the abnormal pancreatic lesions (brown staining), but not in the control. Scale bar: 40 μm.

Table 1

Antigens that can be targeted to evalaute cellular ROS levels in tissue immunohistochemistry

Antibody directed against	Readout for ROS	Tissue Fixation	Reference
8-hydroxy-2-deoxyguanosine (8-oxo-dG)	DNA damage	formalin ethanol	3 4
8-nitroguanine	DNA damage	formalin	5, 6
thymidine glycol (TG)	DNA damage	formalin	7
dinitrophenyl (DNP)	protein oxidation	methacam	8, 9
nitrotyrosine	protein oxidation	formalin	10, 11
4-hydroxy-2-noneal (4HNE)	lipid peroxidation	formalin	3, 12
malondialdehyde (MDA)	lipid peroxidation	formalin	13, 14
acrolein (ACR)	lipid peroxidation	paraformaldehyde	15, 16
methyglyoxal (MG)	lipid peroxidation	formalin	17
hexanoyl-lysine (HEL)	lipid peroxidation	formalin	18, 19
crotonaldhyde (CRA)	lipid peroxidation	paraformaldehyde formalin	20 21
7-ketocholesterol (7-KC)	lipid peroxidation	N/A[*]	22, 23

[*] forzen tissue section

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