Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINDS-AIREN)の診断基準を用いた．17年年間の追跡期間中に349例が認知症を発症し，そのうち316例（90.5%）の脳を剖検あるいはCT/MRIで形態学的に調べた（182例を剖検で，134例を画像診断で評価した）．認知症発症例の病型別内訳はAD162例，VD92例，その他95例だった．

（倫理面の配慮）

本研究は，「疫学研究に関する倫理指針」「ヒトゲノム・遺伝子解析研究に関する倫理指針」に基づき研究計画書を作成し，九州大学医学部倫理委員会の承認を得て行われた．本研究は，すべての対象者からインフォームドコンセントを取得したうえで実施した．研究者は，対象者の個人情報の漏洩を防ぐうえで細心の注意を払い，その管理に責任を負っている．

C. 研究結果

調査開始時に行った運動習慣に関するアンケート調査で，運動習慣があると回答したものを「運動あり群」と定義し，運動習慣の有無と認知症発症との関係を検討した．運動なし群を基準にして，運動あり群におけるADおよびVD発症の相対危険度を求めた。その結果，性，年齢を調整した運動あり群のAD発症の相対危険は0.7と発症リスクが有意に低かった(p=0.04)。運動あり群のAD発症相対危険は，学歴，収縮期血圧，心電図異常（左室肥大，ST低下，心房細動），糖尿病，脳卒中既往，body mass index（BMI），血清総コレステロール，喫煙，飲酒を調整した後でも0.69(p=0.04)と有意であった（図1-a）．VD発症の相対危険度は，運動あり群で0.73と低下傾向を示したものの，統計学的には有意ではなかった（図1-b）．

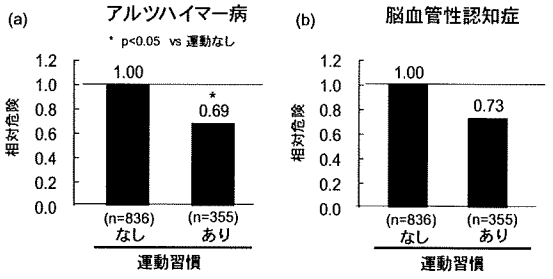

図1 運動習慣の有無別にみた認知症発症の相対危険
(a) アルツハイマー病 (b)脳血管性認知症
久山町男女1,191名，60歳以上，1988-2005年，多変量調整

調整因子：年齢，学歴，収縮期血圧，心電図異常，糖尿病，血清総コレステロール，脳卒中の既往，body mass index，喫煙，飲酒

D. 考察

運動習慣の有無がADおよびVD発症に及ぼす影響について，地域在住高齢者を対象に検討を行った。今回の成績は，運動習慣を有することがAD発症に対して保護的に働くことを示唆している．ADの発症機序として，アミロイドβ蛋白（amyloidβ-protein：Aβ）の異常な凝集や沈着から神経細胞死に至るというアミロイド仮説が最も有力な説として知られている．運動がどのようにAD発症に抑制的に働くのか，そのメカニズムは十分には解明されていないが，持久的なトレーニングにより，ADモデルマウスの脳内のAβ42が減少することが報告されている．また，運動は脳のさまざまな領域において，神経の発生や生存，シナプス可塑性に重要な働きをする脳由来神経栄養因子(Brain-derived neurotrophic factor; BDNF)や神経成長因子(Nerve Growth Factor; NGF)，インスリン様増殖因子I (Insulin-like Growth Factor I; IGF-1)の合成を高めることも多くの動物実験により明らかにされている．したがって，運動はAβの蓄積を抑制するとともに，神経栄養因子を増加させることで神経毒

や酸化ストレスから神経細胞を保護し,AD 発症に抑制的に作用するという可能性が推考される.

近年、ADやVDの危険因子の一つとして耐糖能異常/糖尿病がその発症に関与していることが報告されている．一方で,運動は AMP activated protein kinase (AMPK)を活性化し,グルコース取り込みや脂肪酸の酸化を亢進させることがすでに明らかになっており,運動が糖・脂質代謝の改善に有効であることはよく知られている．したがって,運動は脳に直接的に働きかけるだけでなく,糖・脂質代謝の改善といったAD発症の危険因子の改善を介しても間接的に予防因子として作用するものと考えられた.

E. 結論

運動習慣を有することは、一般地域住民のAD発症の予防因子であった.今後,運動量とAD発症との関連性に加え，AD 発症の危険因子であるAPOE遺伝因子と運動との相互作用を含め,より詳細な検討を行っていく予定である.

G. 研究発表

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H. 知的所有権の取得状況

1. 特許取得　なし
2. 実用新案登録　なし

I. 研究協力者

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別紙４

研究成果の刊行に関する一覧表

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Chapter 13

Quantitative Analysis of Oxidized Guanine, 8-Oxoguanine, in Mitochondrial DNA by Immunofluorescence Method

Mizuki Ohno, Sugako Oka, and Yusaku Nakabeppu

Abstract

8-Oxoguanine (8-oxoG), an oxidized form of guanine, is one of the major mutagenic lesions generated under oxidative stress. Oxidative damage in mitochondrial DNA has been implicated as a causative factor for a wide variety of degenerative diseases as well as for cancer during aging. We established a quantitative method for in situ detection of 8-oxoG in mitochondrial DNA in a single-cell level using a monoclonal antibody. Specific detection of 8-oxoG in mitochondrial DNA was confirmed by pre-treatment of samples with DNase I or MutM, the latter excising 8-oxoG opposite C in DNA. We then analyzed 8-oxoG dynamics in mitochondrial DNA of the wild-type and 8-oxoG DNA glycosylase (OGG1)-deficient mouse cells after exposure to hydrogen peroxide. Intensities for the 8-oxoG immunoreactivity in mitochondrial DNA were increased immediately after the exposure to hydrogen peroxide in both types of cells. The increased intensities returned to basal levels within a few hours only in wild-type cells, but not in OGG1-deficient cells which exhibited the increased intensities even 24 h after the exposure. These results indicate that OGG1 is a major enzyme for excision repair of 8-oxoG in mitochondrial DNA in mouse cells, and that our method described here is appropriate to study 8-oxoG dynamics in mitochondrial DNA.

Key words: 8-oxoguanine, OGG1, immunodetection, immunofluorescence, mitochondrial DNA, oxidative stress, hydrogen peroxide, base excision repair.

1. Introduction

Reactive oxygen species (ROS), generated as byproducts of mitochondrial respiration or as a consequence of exposure to environmental agents, are known to oxidize DNA. Oxidative damage to cellular DNA often causes mutagenesis as well as programmed cell death. The former may result in carcinogenesis, while the latter has been linked to degenerative disorders *(1–3)*. Among many classes

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

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of DNA damage caused by ROS, an oxidized form of guanine base, 8-oxoguanine (8-oxoG) is considered to play a major role in mutagenesis and carcinogenesis, because 8-oxoG can pair with adenine as well as cytosine.

8-OxoG accumulates in both nuclear and mitochondrial DNA (mtDNA) during aging, and the level of accumulation is known to increase in patients with various neurodegenerative diseases, such as Parkinson's disease (PD) or Alzheimer's disease (AD) *(4, 5)*, suggesting that an accumulation of 8-oxoG in cellular DNA contributes to cell death. We recently demonstrated that accumulation of 8-oxoG in mtDNA causes mitochondrial dysfunction and Ca^{2+} release followed by the activation of calpain, resulting in cell death. It is thus important to quantitatively evaluate the levels of 8-oxoG in mtDNA in order to understand the cellular response to the oxidative stress in mitochondria. To this end, we have developed several methods to detect and quantify levels of 8-oxoG in cellular DNA, based on a separation of 8-oxo-2′ -deoxyguanosine in enzyme-digested DNA preparation from cells or tissues on a liquid chromatography with electrochemical detection or MS/MS analysis or in situ immunological detection of 8-oxoG in cultured cells or various tissues derived from mouse or human *(6–9)*.

We herein describe an in situ detection of 8-oxoG in mtDNA in cultured cells or mouse brain sections, using a monoclonal antibody against 8-oxoG (N45.1). In order to ensure the specific and accurate detection of 8-oxoG with the monoclonal antibody, we developed specific pre-treatments. Because the antibody used here rather selectively reacts with 8-oxoG in single-stranded DNA, and it also efficiently reacts with 8-oxoG in RNA, it is required to remove RNA from samples and denature DNA prior to reaction with the primary antibody against 8-oxoG (**Table 13.1**). However, different conditions are required for the denaturation of nuclear and mitochondrial DNA probably because of differences in DNA packaging. We therefore have carefully examined the effects of various combinations of pre-treatment with RNase A or DNase I and denaturation with HCl or NaOH, on in situ detection of 8-oxoG in mitochondrial DNA, and the results are summarized in **Table 13.1** and **Fig. 13.1**. We found that the pre-treatment with RNase A and denaturation with NaOH give clear and specific cytoplasmic immunofluorescent signals for 8-oxoG which are co-localized with signals from MitoTracker (**Fig. 13.1A**) or with immunofluorescent signals from mitochondrial transcription factor A (TFAM) which binds to mitochondrial DNA (**Fig. 13.1B**)*(10)*. When cells are exposed to hydrogen peroxide, cytoplasmic immunofluorescent signals for 8-oxoG detected after RNase A pre-treatment and NaOH-denaturation are dramatically increased in comparison to control cells, and those signals are diminished by either pre-treatment with MutM (an enzyme excising 8-oxoG opposite cytosine in DNA, *see* **Note 1**)

Table 13.1
Combination of nucleases and denaturation methods on the detection of 8-oxoG using the monoclonal anti-8-oxoG antibody, N45.1

		Pretreatment with nuclease			
		None	RNase A	DNase I	RNase A + DNase I
Denaturation	None	rRNA+ Cytoplasmic RNA	weak cytoplasmic signal	rRNA+ Cytoplasmic RNA	No signal
	HCl	rRNA+ Cytoplasmic RNA	Nuclear DNA	rRNA+ Cytoplasmic RNA	No signal
	NaOH	N.D	Mitchondrial DNA	poor morphology	poor morphology

The signal patterns of the immunofluorescence for intracellular 8-oxoG detected by the combination of each pretreatment with nuclease and denaturation method are summarized.

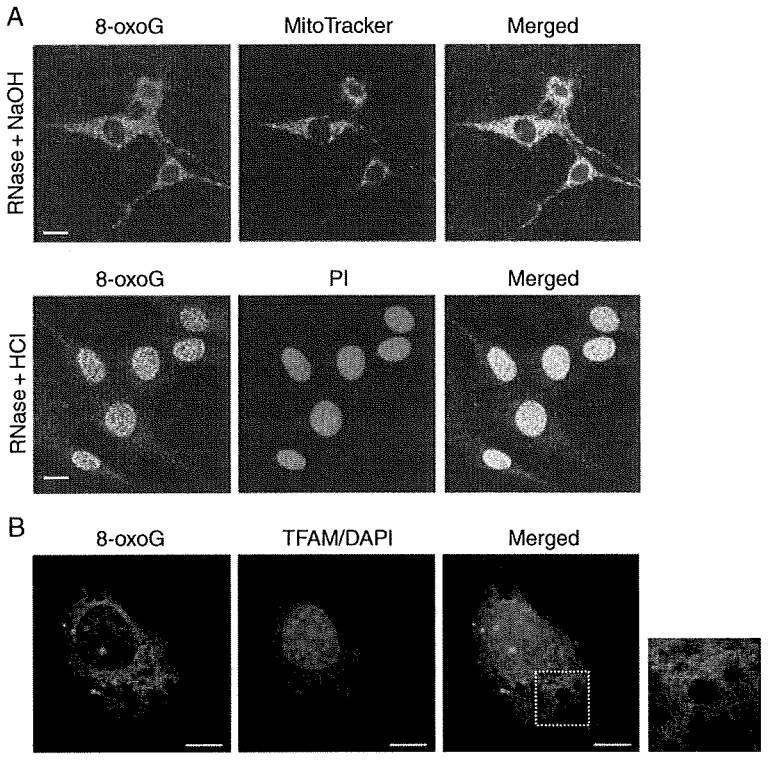

Fig. 13.1. **Immunodetection of 8-oxoG in mitochondrial DNA.** A. Immunofluorescence detection of intracellular 8-oxoG in NIH3T3 cells. *Top panels*: cytoplasmic 8-oxoG immunoreactivity is detected in a sample pre-treated with RNase A and denatured with NaOH (RNase + NaOH). Mitochondria are stained with MitoTracker. *Bottom panels*: nuclear 8-oxoG immunoreactivity is detected in a sample pre-treated with RNase A and

or with DNase I (**Fig. 13.2**). Cytoplasmic immunofluorescent signals for 8-oxoG increase after the exposure to oxidative stress, then return to the basal level within a few hours in wild-type MEFs, however, those in OGG1-null MEFs remain high even 24 h after the exposure (**Fig. 13.3**). From these experiments, we conclude that the denaturation with NaOH after RNase A treatment is the

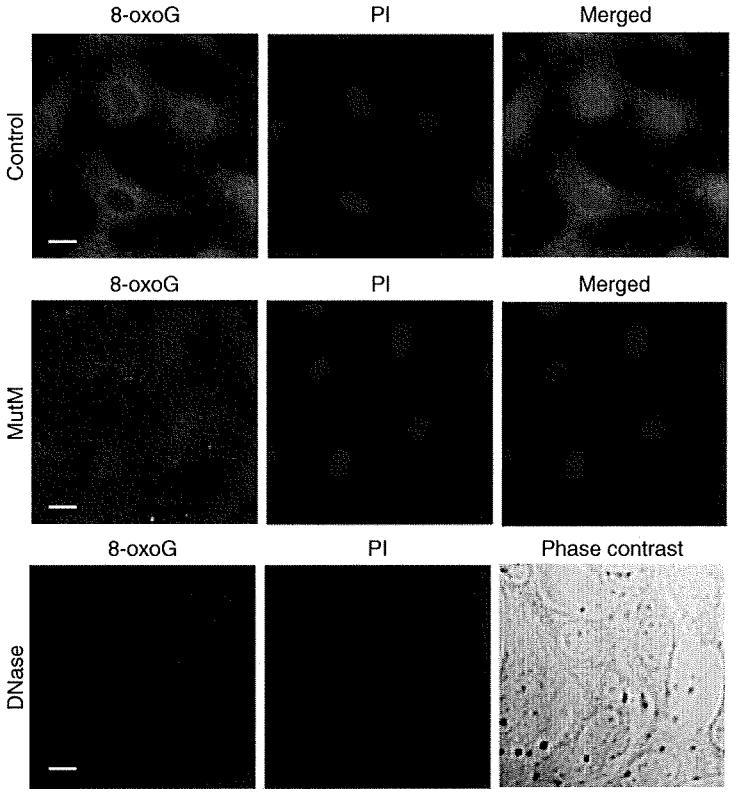

Fig. 13.2 **Cytoplasmic 8-oxoG immunoreactivity detected in a sample pre-treated with RNase A and denatured with NaOH is diminished by MutM or DNAse I treatment.** NIH3T3 cells are cultured on a slide glass. Cells are exposed to hydrogen peroxide for 30 min prior to fixation. Increased 8-oxoG immunoreactivity in cytoplasm after exposure to H_2O_2. Control sample is pre-treated with RNase and denatured with NaOH. Cytoplasmic signals of 8-oxoG immunoreactivity are largely diminished by the additional pre-treatment with MutM (MutM). Cytoplasmic signals of 8-oxoG immunoreactivity are completely diminished by the additional pre-treatment with DNase I (DNase). Although there is no PI signal, cells remained intact (phase contrast).

Fig. 13.1 (continued) denatured with HCl (RNase + HCl). Nuclear DNA is stained with propidium iodide (PI). Bar: 10 μm. B. Cytoplasmic 8-oxoG immunoreactivities were co-localized with mitochondrial transcription factor A (TFAM) in Nu-hOGG1 MEFs which were exposed to 50 μM menadione for 1 h. A part of the merged image was magnified. Nuclei were counterstained with DAPI. Bar: 10 μm. (Adapted from **Ref. *(12).***)

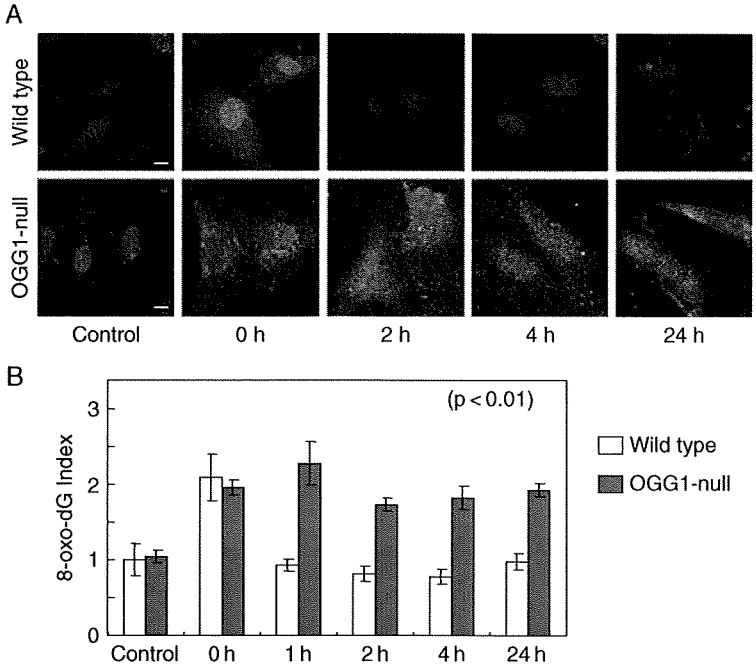

Fig. 13.3. **Dynamics of 8-oxoG immunoreactivity in mitochondrial DNA after exposure of wild-type and OGG1-null MEFs to hydrogen peroxide.** MEFs are exposed to H_2O_2 for 30 min, and at given time cells are subjected to 8-oxoG immunofluorescence microscopy with RNase pre-treatment and NaOH denaturation. Control: no exposure to H_2O_2. Nuclei were counterstained with propidium iodide. Bar: 20 μm. 8-OxoG immunoreactivity in mtDNA increased immediately after exposure to H_2O_2 (0 h), and the levels in wild-type cells were greatly reduced within 2 h after the exposure and returned to the basal level thereafter (*top panels*), while those in OGG1-null cells were not reduced even 24 h after the exposure and remained high (*middle panels*). B. 8-OxoG index in mtDNA was determined and plotted as a bar graph. $p<0.01$, repeated measurement ANOVA.

most suitable pre-treatment to detect 8-oxoG in mitochondrial DNA of cultured cells. To detect mitochondrial 8-oxoG in mouse brain sections, the denaturation step with NaOH should be avoided because the treatment causes disruption of tissue structure resulting in unreproducible results *(9)*.

2. Materials

2.1. Cell Lines

1. NIH3T3 cells *(11)*.
2. Wild-type and *Ogg1*$^{-/-}$ mouse embryo fibroblasts (MEFs). The generation of *Ogg1*$^{-/-}$ mice has been described previously *(12)*. *Ogg1*$^{-/-}$ and wild-type MEFs are isolated from embryos that were obtained by mating *Ogg1*$^{+/-}$ female and male mice.

3. Nu-hOGG1 MEFs: *Ogg1*$^{-/-}$ MEFs immortalized as above were transfected with a vector pcDNA3.1Hyg(−):hOGG1-1a encoding the nuclear form of hOGG1 *(13)*.

2.2. Cell Culture

1. Growth medium A: Dulbecco's Modified Eagle's Medium (DMEM, low glucose, GIBCO 11885) supplemented with 10% calf serum (CS, GIBCO 200-6170A), penicillin–streptomycin (GIBCO 15140).
2. Growth medium B: Dulbecco's Modified Eagle's Medium (DMEM, high glucose, GIBCO 11995-065) supplemented with 10% fetal bovine serum (FBS, GIBCO), penicillin–streptomycin (GIBCO 15140).
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA, 1 mM, IBL 23315).
4. Phosphate-buffered saline: Dissolve five tablets of PBS (Dulbecco's 28-103-05 FH) in distilled water, make up to 1,000 ml, autoclave.
5. Poly-L-lysine coated glass slides (thickness 0.9–1.2 mm, precleaned, 76 × 26 mm): Before starting the culture, prepare culture glasses as follows. Set pre-cleaned slide glasses to the slide holder, wrap with aluminum foil, and autoclave. Put sterile slide glass into the culture dish. Culture cells on slide glass for immunological staining.
6. MitoTracker Red (Molecular Probes M-7512).
7. Medium containing hydrogen peroxide: 0.5 mM hydrogen peroxide (H_2O_2) in serum -free DMEM. Prepare medium containing H_2O_2 freshly for each experiment. Use within 30 min of preparation.
8. 2-Methyl-1.4-naphthoquinone (menadione) is dissolved in PBS at 500 mM, stored in single-use aliquots at –30°C. Dilute with low glucose DMEM without FBS and penicillin–streptomycin and filter through 0.2-μm filter (Millipore, Corp., Bedford, MA, USA) for use.
9. Fixative: cold methanol (100%) stored at –20°C.
10. Paraformaldehyde (PFA, Nacalai tesque, for electron microscopic grade): 4% (w/v) in PBS, use fresh solution for each experiment. PFA should be handled under the hood because of toxicity. Transfer PFA powder to sterile bottle, add PBS to make 4% solution, put magnetic stir bar in the bottle, cap, heat bottle with stirring at 60°C for 2–h until completely dissolved (use water bath with magnetic stirrer).

2.3. Immunological Detection of Cellular 8-OxoG

1. Anti-8-oxoG monoclonal antibody (clone N45.1, Nikken SEIL Co., Ltd).
2. Goat polyclonal anti-mitochondrial transcription factor A (TFAM) antibody (SC-23588 Santa Cruz).

3. RNase solution: Stock solution; 10 mg/ml RNase A (Sigma) in 10 mM Tris–HCl (pH 7.5), 15 mM NaCl. To inactivate contaminating DNase, incubate RNase solution at 98°C for 15 min, then it leave at room temperature until cool. Store at –20°C until use. Working solution: 1/2 dilution of stock solution with same buffer.
4. DNase solution: 1 U/μl of DNase I (Sigma) in 50 mM Tris–HCl (pH 7.5), 0.1 mM $MgCl_2$.
5. MutM solution: 10 μg/ml of MutM protein (Fapy DNA glycosylase, Sigma) in nicking buffer [10 mM Tris–HCl (pH 7.5), 5 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 100 μg/ml BSA] (*see* **Note 1**).
6. HCl solution: 2 N HCl in distilled water. Prepare freshly.
7. NaOH solution: 50 mM NaOH in 50% ethanol. Prepare freshly.
8. Tris-base neutralization solution: 50 mM Tris-base in distilled water.
9. Blocking solution: 1X BlockAce (Dainippon Pharmaceutical, Japan).
10. Dilution solution for antibodies: 1/10 dilution of BlockAce with distilled water.
11. Wash solution: PBD:PBS + 0.1% Triton-X 100.
12. Secondary antibody: anti-mouse IgG Alexa-488 conjugate (Molecular Probes).
13. Counter staining solution (Propidium Iodide solution, PI, Sigma): Stock solution: 1.5 mM (1 mg/ml) PI in water, store at 4°C. Working solution: diluting the stock solution 1:3,000 in PBS (500 nM). DNA and RNA are both stained with PI.
14. Anti-fade mounting media for fluorescence microscopy: Vectashield (Vector Lab) is used to prevent rapid fluorescence quenching during microscopic examination.

2.4. Immunodetection of Mitochondrial 8-OxoG in Mouse Brain Section

1. Sucrose solutions: Prepare 20% and 30% sucrose (w/v) in PBS.
2. Disposable plastic dish for tissue freezing (Cryomold, Sakura).
3. Tissue freezing medium (O.C.T compound, Sakura).
4. 6-Well culture dish (Nunc).
5. Painting brush: A small and soft painting brush is useful to pick up free-floating tissue section from solution without damage.

6. DAPI solution for nuclear DNA staining: Prepare stock solution (0.5 mg/ml), keep at 4°C, avoiding light.
7. Rabbit anti-VDAC polyclonal antibodies were described previously *(10)*.
8. Other solutions required are as above (**Section 2.3**).

2.5. Microscope and Software

1. A fluorescence microscope Axioskop2 equipped with a CCD camera AxioCam (Carl Zeiss).
2. Digital image processing and camera control: AxioVision software (Carl Zeiss).
3. Images processing: Adobe Photoshop 7.0 Software (Adobe systems, Inc).

3. Methods

3.1. Cell Culture

1. NIH3T3 cells are maintained in growth medium A, with 5% CO_2 at 37°C. The cells on culture dishes are treated with trypsin–EDTA solution and passaged once per 3 days (1:3).
2. MEF culture: Primary MEFs are maintained in growth medium B, with 5% CO_2 at 37°C.
3. When primary culture of MEFs reaches 90% confluence, cells on culture dishes are treated with trypsin–EDTA solution and passaged (1:3).
4. For immunofluorescent microscopy, cells are directly cultured on poly-L-lysine coated glass slides. Put a sterile slide glass on a 100-mm culture dish, add 5 ml of pre-warmed growth medium to cover the surface of slide glass with medium, and add 2–5 $\times$ 10^5 cells in 5 ml of growth medium to the culture dish with slide glass, gently mix, and incubate in the CO_2 incubator.
5. When multiple cell slides are necessary, a sterile square dish (sterile no. 2 square scale, Eiken Chemical Co., Ltd) can be used with five slides, add 5 ml of pre-warmed growth medium, then 0.8–1.2 $\times$ 10^6 cells in 20 ml of growth medium are added.
6. The cells are cultured for 16 h on slide glasses and then are used for the experiments.
7. For mitochondrial staining, medium is changed with freshly prepared medium containing 400 nM MitoTracker Red, and cells are incubated 45–60 min in the CO_2 incubator. Remove the medium from the culture by aspiration, and add gently 6 ml of pre-warmed complete growth medium per 100-mm

dish. Incubate the culture at 37°C for 5 min to allow unbound MitoTracker to diffuse into the medium. Repeat this procedure once before fixation.

8. Fixation: Remove the medium by aspiration, and rinse cells with cold PBS (4°C), transfer slides to cold methanol, fix for at least 10 min at –20°C. Slides can be kept in methanol for a week at –20°C.

3.2. Hydrogen Peroxide Exposure

1. The cells cultured on a slide glass are gently rinsed with a pre-warmed serum-free medium, and then are incubated with 0.5 mM of H_2O_2 in a serum-free medium for 30 min at 37°C (*see* **Note 2**).
2. After 30 min, the medium is removed by aspiration, and then cells are incubated in pre-warmed complete medium containing serum. After the 30-min exposure to H_2O_2, cells are fixed at 0, 2, 4, and 24 h later.
3. The slides are washed three times with cold PBS at given time.
4. Fix slides with cold methanol for at least 10 min. Slides are kept in methanol at –20°C until use.

3.3. Pre-treatment for Immunodetection of 8-OxoG in Mitochondrial DNA

1. Fixed slides with methanol are rinsed with PBS twice at room temperature (RT).
2. To eliminate RNA, the slides are treated with RNase solution for 1 h at 37°C (*see* **Note 3**).
3. Rinse the slides with PBD three times, 3 min for each, at RT. Transfer slides into PBS.
4. To obtain the negative control or confirm specificity of the immunoreactivity, pre-treatment with MutM or DNase I is performed. Incubate slides with MutM solution or DNase solution for 1 h at 37°C. Then slides are rinsed with PBD three times, 3 min for each, at RT (refer **Fig. 13.2**).
5. Slides are incubated with 4% PFA for 10 min at RT to re-fix cells, then rinsed with PBD three times, 3 min for each, at RT (*see* **Note 4**).
6. Denaturation of mitochondrial DNA: Dip slides into NaOH solution for 7 min at RT, then rinse with PBS three times, 3 min for each, at RT.

3.4. Pre-treatment for Immunodetection of 8-OxoG in Nuclear DNA

1. Fixed slides with methanol are rinsed with PBS twice at RT.
2. To eliminate RNA, slides are treated with RNase solution for 1 h at 37°C.
3. Rinse slides with PBD three times, 3 min for each, at RT. Transfer slides to PBS.
4. To obtain the negative control or confirm specificity of the immunoreactivity, pre-treatment with MutM or DNase I is

performed. Incubate slides with MutM solution or DNase solution for 1 h at 37°C. Then slides are rinsed with PBD three times, 3 min for each, at RT.

5. Denaturation of nuclear DNA: Rinse slides with distilled water, then dip the slides into 2 N HCl for 10 min.
6. Dip slides into Tris-base solution for 10 min.
7. Rinse slides with PBS for three times, 3 min for each, at RT.

3.5. Immunofluorescence Staining

1. Pre-treated slide is placed in blocking solution prepared in a Coplin Jar for 10 min at RT to reduce non-specific signals.
2. Diluted mouse anti-8-oxoG monoclonal antibody (1:10 in dilution solution), with appropriately diluted other antibodies if necessary, is applied to the slide, which is then covered with a plastic cover slip and incubated for 2 h at RT in a dark, humidified chamber.
3. Rinse the slide with PBD three times, 3 min each, at RT with gentle shaking.
4. Place the slide in blocking solution for 10 min at RT.
5. Diluted secondary antibody (1:300 in dilution solution) is applied to the slide, which is then covered with a plastic cover slip and incubated for 40 min at RT in a dark, humidified chamber.
6. Rinse the slide with PBD three times, 3 min each, at RT with gentle shake.
7. Dip the slide into PI solution for 5 min to stain nuclear DNA at RT, and then rinse the slide with PBD.
8. Mount the slide with anti-fade mounting media. Store the slide at 4°C until observation under the microscope.

3.6. Fluorescent Microscopy and Image Analysis

1. Fluorescent images are observed using an Axioskop2 equipped with an AxioCam. A digital image of each fluorescent signal is captured separately as a gray-scale image. Merging of the images with different fluorescences is performed using the AxioVision software. Captured images are processed using Adobe Photoshop 7.0 software.
2. To obtain an 8-oxoG index, each image of 8-oxoG immunofluorescence is converted to 256-level gray-scale, whereas each image of nuclear PI staining is converted to a binary monochrome using the Adobe Photoshop.
3. From the gray-scale image of 8-oxoG immunofluorescence, the signals present in areas corresponding to nuclei represented by the monochrome PI image are deleted, and then the image with only cytoplasmic signals for 8-oxoG immunofluorescence is saved as a tiff file.

4. The tiff file of image with the cytoplasmic 8-oxoG immunofluorescence is opened using NIH Image 1.61 software.
5. An area of a single cell is manually defined, and an integrated pixel density for each cell is determined with a uniform threshold.
6. At least 30 cells are analyzed, and an average value of the integrated pixel density per cell is calculated as the 8-oxoG index for each sample.

3.7. Immunodetection of Mitochondrial 8-OxoG in Mouse Brain Section

3.7.1. Sample Preparation

1. Animals deeply anesthetized with pentobarbital (30 mg/kg i.p.) are perfused intracardially with saline followed by cold 4% paraformaldehyde (PFA) in 0.1 M PBS.
2. Brains are removed and fixed in 4% PFA for 12 h at 4°C.
3. Cryoprotection: Rinse fixed brains with 20% sucrose, and transfer to 20% sucrose for 24 h at 4°C. Next, brains are transferred to 30% sucrose for 24 h at 4°C.
4. Rinse brains with PBS twice, remove extra liquid.
5. Cut in half and trim fixed brain, then set it onto the cryomold.
6. Add O.C.T compound and freeze brain immediately to set cryomold on metal block in liquid nitrogen. Keep brain at –80°C until use.
7. Serial sections (40 mm thick) are cut on a cryostat and each section is collected as a free-floating section in a well filled with PBS, and processed immediately for immunodetection.

3.7.2. Detection of 8-OxoG in Mitochondrial DNA in Mouse Brain

To detect 8-oxoG in mtDNA in tissue section, the alkali denaturation step is not recommended because the treatment causes disruption of tissue structure resulting in unreproducible results. Thus, avoid this step *(9)*.

1. Aspirate PBS carefully (do not aspirate floating tissue) from the well, add 500 µl of pre-warmed RNase solution. Incubate for 1 h at 37°C.
2. Aspirate RNase solution carefully, add PBD, wash for 10 min at RT with gentle shake. Repeat this step for three times.
3. Aspirate PBD carefully, add blocking solution, and incubate for 30 min at RT.
4. Aspirate blocking solution carefully, add 1:100 diluted anti-8-oxoG antibody and anti-VDAC antibodies, and incubate for overnight at 4°C.
5. Aspirate antibody solution carefully, add PBD, and wash for 10 min at RT with gentle shake. Repeat this step three times.
6. Aspirate PBD carefully, add 1 ml of blocking solution, and incubate for 30 min at RT.

7. Aspirate blocking solution carefully, add 500 ml of 1:300 diluted proper Alexa Fluor-labeled secondary antibodies, and incubate for 45 min at RT.
8. Aspirate secondary antibody solution carefully, add PBD, and wash for 10 min at RT with gentle shake. Repeat this step three times. Add DAPI solution for last PBD (1:5,000 dilution of stock solution with distilled water).
9. The free-floating sections are picked up using painting brush and set onto the slide glass, mount with Vectashield. Keep slide at 4°C until use.
10. Confocal images (**Fig. 13.4**) are acquired under Eclipse TE300 (Nikon, Japan) equipped with the Radiance 2100 laser scanning confocal microscope system (Bio-Rad Laboratories).

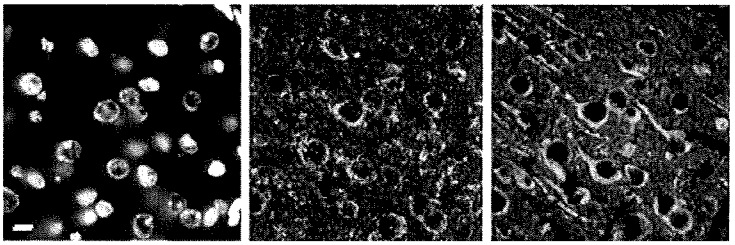

Fig. 13.4. **Immunodetection of mitochondrial 8-oxoG in mouse brain.** The sections were subjected to immunofluorescent staining with the anti-8-oxoG antibody after RNase treatment. Laser scanned confocal images from the cerebral cortex stained for nuclear DNA with DAPI, 8-oxoG, and VDAC are shown. Weak peri-nuclear immunoreactivities for 8-oxoG were observed in large neurons, and these immunoreactivities were mostly co-localized with the mitochondrial protein VDAC immunoreactivity. Scale bar: 10 μm. (Adapted from Ref. *(9)*.)

4. Notes

1. MutM is a bacterial enzyme, 8-oxoG DNA glycosylase that has an activity to excise 8-oxoG opposite cytosine in duplex DNA. This protein is also called FPG (formamidopyrimidine–DNA glycosylase). OGG1 is a functional homolog of MutM.
2. NIH3T3 and MEFs cells are not killed by exposure to H_2O_2 under these conditions (0.5 mM, 30 min). You will see shrinking of cells after 30-min exposure, and then recovery within a few hours.
3. Complete digestion of RNA in the fixed cells is required to obtain a clear immunofluorescent signal. The concentration of RNase A used here is much higher than that in usual

reagents for molecular biological experiments. During handling of this solution, careful attention should be paid to avoid contamination of equipment and other reagents with the RNase. Use humidified chamber, jars, and pipettes prepared only for this treatment. RNase activity on glassware or metalware can be inactivated by dry-oven at 200°C for 2 h.

4. Re-fixation by PFA is necessary only when cells are treated with NaOH.

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