

Chapter 2

Molecular Analysis of Genetic Instability Caused by Chronic Inflammation

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Summary

Genetic instability is a hallmark of human cancers. It is the driving force for tumor development as it facilitates the accumulation of mutations in genes that regulate cell death and proliferation and therefore promotes malignant transformation. Chronic inflammation is a common underlying condition for human tumor development, accounting for approximately 20% of human cancers. TNF α is an important inflammation cytokine and is crucial to the development of inflammation-associated cancers. We have shown that TNF α can cause DNA damages through reactive oxygen species (ROS). TNF α treatment in cultured cells resulted in increased gene mutations, gene amplification, micronuclei formation and chromosomal instability. Antioxidants significantly reduced TNF α -induced genetic damage. In addition, TNF α treatment alone led to increased malignant transformation of mouse embryo fibroblasts, which could be partially suppressed by antioxidants. Therefore, genetic instability plays an important role in inflammation-associated cancers.

Key words: Genetic instability; Inflammation; Cancer; Reactive oxygen species; 8-Oxo-deoxyguanosine.

1. Introduction

1.1. *Inflammation and Cancer*

Exposure to environmental carcinogen and chronic inflammation are two important underlying conditions for sporadic human tumor development. Chronic inflammations predispose patients to cancers. For example, chronic atrophic gastritis increases the risk for gastric cancer, chronic hepatitis for hepatic carcinoma and chronic skin ulcer for squamous cancer of the skin.

Most studies on the mechanism underlying inflammation-associated cancers focused on NF κ B signaling. We recently found that ROS-induced DNA damage and genetic instability is another

important contributing factor in the development of cancers in chronic inflammation (1). ROS is abundant in inflammation. It can be produced by the respiratory burst in the inflammatory cells or induced by inflammatory cytokines such as TNF α . We have found that TNF α induces ROS, causes genetic aberrations and leads to transformation, all of which were at least partially inhibited by antioxidants (1).

1.2. Genetic Assays

In order to study instability in inflammation-associated cancers, a variety of genetic assays can be applied to assess the oxidative stress and DNA damages, which include micronucleus assay, cytogenetic analysis of chromosomal aberrations, gene amplification assay, mutation assay, comet assay and immunostaining for 8-oxodG and γ -H2AX. The malignant transformation resulting from accumulation of mutations can be determined by transformation assays such as soft agar assay. This chapter will elaborate the detailed protocols for these assays.

2. Materials

2.1. Cell Culture

1. 379.2 cells are p53^{-/-} colon carcinoma HCT116 cells, which were kindly provided by Dr. Bert Vogelstein of Johns Hopkins University, Baltimore, MD. 379.2 cells were cultured in McCoy 5A medium supplemented with 10% fetal bovine serum. L929 cells were maintained in DMEM medium supplemented with 10% equine serum. 10T1/2 and BALB/3T3 are mouse embryonic fibroblasts obtained from Cell Culture Facility of Duke University Comprehensive Cancer Center (Durham, NC). They were maintained in DMEM medium supplemented with 10% fetal bovine serum.
2. Recombinant human and mouse TNF α were purchased from R&D Systems, Inc (Minneapolis, MN 55413). *N*-acetyl cysteine (NAC), Vitamin C, Vitamin E, actinomycin D, rotenone and dichlorofluorescein diacetate (DCFDA) were purchased from Sigma (St. Louis, MO).

2.2. Gene Amplification Assay

1. The selective agent for *cad* gene amplification, PALA, was obtained from the Drug Synthesis Branch, Division of Cancer treatment, National Cancer Institute.
2. Methotrexate (MTX) was purchased from Sigma (St. Louis, MO).
3. Methylene blue solution: 0.4% methylene blue dissolved in 30% methanol solution.
4. 1% Acetic acid solution: 1% acetate in 50% methanol.

2.3. Analysis of Chromosomal Aberrations

2.3.1. In Vitro Analysis of Chromosomal Aberrations in Cultured Cells

5. Crystal violet (CV) staining fixative: 0.5% CV in 80% methanol.
6. Dissolves PALA/MTX in ddH₂O to make 1,000× stock solution.

1. Colcemid (10 µg/ml) is purchased from Gibco (now Invitrogen) or Irvine Scientific.
2. 0.5% Colchicine (Sigma C3915) in water (see recipe; store at -20°C).
3. 1-cc disposable syringes and 23 gauge needles.
4. 15-ml Conical centrifuge tubes (Corning #25310).
5. Hypotonic solution: 0.075 M KCl (0.56% KCl) in ddH₂O.
6. Fixative: 3 volume of methanol mixed with 1 volume of acetic acid.
7. Giemsa stain solution: (Dissolving one tablet in 100 ml ddH₂O and add 5 ml R66 solution. Both the tablet and R66 solution were purchased from BDH Laboratory Supplies (Poole, England)).

2.3.2. Fluorescence In Situ Hybridization (Suitable for Chromosome Painting)

1. Hybridization mixture: mix 2 ml 20× SSC at pH 5.8 and 10 ml formamide, add 2 g dextran sulfate on the top and vortex to mix. Then leave on bench top over night for dextran sulfate to dissolve. Aliquot and store in -20°C.
2. 20× SCC at pH 5.8 and pH 7.4: Mix thoroughly 175.3 g NaCl, 88.2 g sodium citrate in 800 ml ddH₂O. Adjust pH, adjust volume to 1 L, store at room temperature for up to 6 months.
3. 70% Formamide denaturation solution: 5 ml 20× SCC at pH 5.8 and 35 ml formamide in 10 ml ddH₂O. Store covered between uses. Discard after 2 months.
4. 50% Formamide in 2× SSC: 5 ml 20× SCC at pH 5.8 and 25 ml formamide in 20 ml ddH₂O. Store covered between uses. Discard after 2 months.
5. 2× SSC pH 7.4: 5 ml 20× SCC at pH 7.4 in 45 ml ddH₂O. Discard after use.
6. PN buffer: Use 0.1 M NaH₂PO₄ to adjust pH of the 0.1 M Na₂HPO₄ to achieve pH 8.0 and add 0.05% Nonidet® P-40. Autoclave and store up to 6 months.
7. For chromosome painting, whole chromosome probes for mouse chromosomes were purchased from Cambio Ltd, Cambridge, UK.

2.4. Detection of Aneuploid Cells by FACS Analysis

1. Wash buffer: PBS + 1% fetal bovine serum (FBS).
2. Fixative: cold 70% ethanol.
3. Staining buffer: propidium iodide (10 µg/ml) and ribonuclease A (100 µg/ml) in PBS.

4. Flow cytometry machine and ModFit LT cell-cycle analysis software (Verity Software House, Topsham, ME).

2.5. Micronucleus Assay

1. Cytochalasin B (Sigma, St. Louis, MO, USA) stock solution: 5 mg/ml dissolved in ethanol.
2. Carnoy fixative (ratio of volume: methanol:acetic acid = 3:1).
3. 2×SSC buffer with NP40: 2× sodium chloride–sodium citrate (SSC) buffer with 0.1% Nonidet® P 40 (NP 40).
4. Acridine orange (Sigma-Aldrich) staining buffer: 5–10 µg/ml acridine orange (AO) dissolved in 0.1 M sodium phosphate (pH 7.2) + equal volume of heptane.
5. Fluorescence microscope with FITC filter.

2.6. Immunodetection for 8-oxodG in Mouse Tissues

1. Fixative: acetone: Methanol (1:1).
2. M.O.M.™ Immunodetection Kit FLUORESCCEIN (Catalog No. FMK-2201) is purchased from Vector Laboratories, Inc (Burlingame, CA 94010).
3. M.O.M.™ Mouse Ig Blocking Reagent: add two drops of stock solution to 2.5 ml of PBS.
4. M.O.M.™ Diluent: add 600 µl of protein concentrate stock solution to 7.5 ml of PBS.
5. M.O.M.™ Biotinylated Anti-Mouse IgG Reagent: add 10 µl of stock solution to 2.5 ml of M.O.M.™ diluent prepared above.
6. VECTOR ABC Reagent: add two drops of Reagent A to 2.5 ml PBS, mix and then add two drops of Reagent B and mix. Allow ABC Reagent to stand for 30 min prior to use.
7. DAB Enhancing Solution (cat# H-2200) or VECTOR VIP Substrate Kit (cat# SK-4600).

2.7. Soft Agar Assay and Tumorigenesis Assay

1. 10× agar stock: Boil 5% (w/v) bacto-agar (DIFCO, Detroit, MI) stock in distilled water, autoclave to sterilize, and store in 50-ml aliquots at room temperature.
2. Cell culture medium as appropriate for each cell line.
3. 6- to 8-week-old athymic nude mice were purchased from Charles River Laboratories, Raleigh, NC.

3. Methods

3.1. Gene Amplification Assays

Standard gene amplification measures the frequency of amplification of *cad* or *dhfr* gene. *cad* gene encodes carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase. *dhfr*

gene encodes dihydrofolate reductase. The sole known mechanism for PALA resistance is amplification of *cad* gene, therefore frequency of *cad* amplification can be quantified by clonogenic assay with PALA selection. Similarly, MTX can be used to quantify the frequency of *dhfr* gene amplification (2).

1. 50% Lethal dosage (LD_{50}) of the drug has to be determined to decide the concentration of PALA or MTX for each cell line. (1) Seed $2-10 \times 10^3$ cells/well depending on the cell size (refer **Subheading 4**) into a 24-well plate; (2) Cells are grown in the presence of a concentration titration of PALA/MTX until the well of cells grow to confluence in the absence of PALA/MTX; (3) Wash cells once with PBS, then stain cells with methylene blue solution for 20 min; (4) Wash cells with distilled water 3–4 times then extract methylene blue with 1 ml 1% acetic acid solution; (5) Measure the absorbance OD₆₅₀ at $\lambda = 650$ nm then make a graph to determine LD_{50} , the concentration that inhibited 50% cell growth (3, 4).
2. For estimation of resistant clones, about $1-2 \times 10^6$ cells were seeded into each P10 dish (10-cm dish). Cells were then selected in $3.5-9 \times LD_{50}$ of PALA or MTX. Medium should be changed with fresh PALA/MTX every 3–5 days (*see* **Notes 1 and 2**).
3. At the same time when seeding the cells, seed 200–500 cells into at least three plates and let the cells grow in the absence of selective drugs for calculation of plating efficiency (PE). $PE = \text{number of colonies formed} / \text{number of cells seeded}$ (*see* **Note 3**).
4. Resistant colonies usually appear in 2–3 weeks. Cells were fixed and stained with 0.5% crystal violet in 80% methanol. Colonies were counted subsequently (*see* **Note 4**).
5. Frequency of amplification is expressed as the number of resistant colonies relative to the number of colonies formed without PALA and MTX (2, 5).

3.2. Analysis of Chromosomal Aberrations

3.2.1. In Vitro Analysis of Chromosomal Aberrations in Cultured Cells

1. Colcemid® is added to the cell culture at a final concentration of $0.02 \mu\text{g/ml}$. Harvest the cells by 5 min centrifuge at $400 \times g$ 1–4 h later depending on how rapidly the cells grow (*see* **Note 5**).
2. Cells are resuspended in 10 ml 0.075 M KCl and incubated at 37°C water bath for 10–15 min (*see* **Note 6**).
3. 2 ml Fixative is added and mixed well at the end of incubation.
4. Cells are spun down and the supernatant is removed. Cells are resuspended in 10 ml fixative at room temperature.
5. **Step 4** is repeated once.

6. Cells are then collected by 5 min centrifuge at $400 \times g$ and suspended in 0.5–2 ml fresh fixatives depending on the number of cells (addition of fixative should be just enough to make a thin cell suspension, solution in tube will look slightly opaque) (*see Note 7*). Cell suspension is then dropped onto cold wet slides. Cell concentration and cell spreading are monitored by phase contrast microscopy (*see Note 8*).
7. Slides are stained with 0.5% Wright stain or Giemsa stain for 10 min. Metaphase spreads are examined under oil immersion microscope for chromosomal aberrations including chromosome breaks, rings, dicentrics, terminal deletions and interstitial deletions (double minutes). The number of chromosome in each spread can be counted under a microscope to assess the abnormality in ploidy.

3.2.2. *In Vivo Analysis of Chromosomal Aberrations in Mouse Bone Marrow Cells*

This protocol was modified from the one published on The Jackson Laboratory website (6).

1. Inject mouse with 0.1 cc of 0.5% colchicine (stock solution) intraperitoneally. Wait 30–60 min depending on the age of the mice, shorter for young mice and longer for old ones.

2. Sacrifice mouse and remove femur(s) and tibia(s).

Early metaphases seem to be more prevalent in tibias.

3. Cut off just enough of the bone heads to insert a 23 gauge needle into the marrow cavity.
4. Flush out cells into a conical centrifuge tube using a 1-cc syringe filled with 0.075 M KCl.
5. Incubate the tubes at 37°C for 10–15 min.
6. Centrifuge at $400 \times g$ for 5 min in a clinical bench-top centrifuge.
7. Remove supernatant and add 0.5 ml of fixative without disturbing the pellet. Remove fixative after 3–4 s and add 2 ml fresh fixative without disturbing the pellet.
8. Allow tubes to sit at room temperature 30 min.

The procedure can be interrupted at this point and resumed later. Always refrigerate cells if they are to be left standing in fixative longer than 30 min

9. After 30 min centrifuge the cells at $400 \times g$, remove the fixative, and resuspend the cells in fresh fixative.
10. Repeat **step 9** once.
11. Continue as described in **steps 6** and **7** in **Subheading 3.2.1**.

3.2.3. *Fluorescence In Situ Hybridization (Suitable for Chromosome Painting)*

This protocol was modified from that published by Lichter et al. (7) and can be used for chromosome painting to study chromosome translocations.

Pretreatment of
Chromosome Slides

1. Apply 20 μ l of 100 μ g/ml RNase to the target region of the slides at 37°C for 3 min.
2. Wash two times for 3 min in 2 \times SSC.
3. Place slides in 0.01 N HCl containing 100 mg/L pepsin at 37°C for 5 min (*see Notes 9–11*).
4. Wash two times for 5 min in PBS.
5. Dehydrate the slides by placing in 70, 90, and 100% ethanol for 1 min each at room temperature.
6. Air-dry the slides and proceed to slide denaturation. Slides may be used immediately or store at –20°C.

Denaturation of
Chromosome Slides

1. Denature the slides for 2 min in 70% formamide denaturation solution at 70°C. For old slides, denature extra 6 s for every 1 month of slides stored.
2. Immediately transfer slides to ice-cold 70% ethanol for 2 min to reduce strand reannealing before adding the probe.
3. Dehydrate slides by placing in 70, 90, and 100% ethanol for 2 min each at room temperature.
4. Allow slides to air-dry for a few minutes.
5. Prewarm slides to 37°C in an incubator.

Probe Denaturation and
Hybridization

1. Mix thoroughly 0.5 μ l fluorochrome labeled probe (~100 ng), 9 μ l Hybridization mixture, 2 μ l of Cot-1 DNA (~2 μ g), and 3.5 μ l water.
2. Denature the probe by incubating at 82°C for 10 min (this may be done in a PCR machine for convenience) (*see Note 12*).
3. Prehybridization of the probe mixture with excess unlabeled genomic or Cot-1 DNA (usually at least 20 times the amount of the probe DNA) for 30 min to 1 h at 37°C is necessary to reduce the diffuse hybridization of repetitive sequences in the probe to multiple chromosome sites.
4. Spin briefly to collect probe cocktail.
5. Apply the 15 μ l denatured and prehybridized probe cocktail onto the denatured chromosome slide and overlay with a 22 \times 22 mm coverslip.
6. Seal the coverslip with rubber cement to prevent evaporation during hybridization.
7. Place slides in a pre-warmed dark box and incubate 16–18 h at 37°C. Depending on the concentration and complexity of the probe, hybridization time may vary from overnight to several days without detrimental effects (*see Note 13*).

Posthybridization Washing Steps

1. Carefully remove rubber cement. Do not remove the coverslips.
2. Place slides 4 min each in two jars containing prewarmed 50% formamide in 2× SSC at 42°C. The coverslips will come off (*see Note 14*).
3. Place slide 4 min each in two jars containing prewarmed 2× SSC at 42°C.
4. Place slides 4 min each in two jars containing prewarmed PN buffer at 42°C.

Detection

1. Do not allow the slides to dry after the washing steps.
 2. Apply 15 μl DAPI counterstain and antifade solution to the target area of the slides and apply coverslip.
 3. Perform microscopic analysis.
- See Fig. 1* for an example of the FISH.

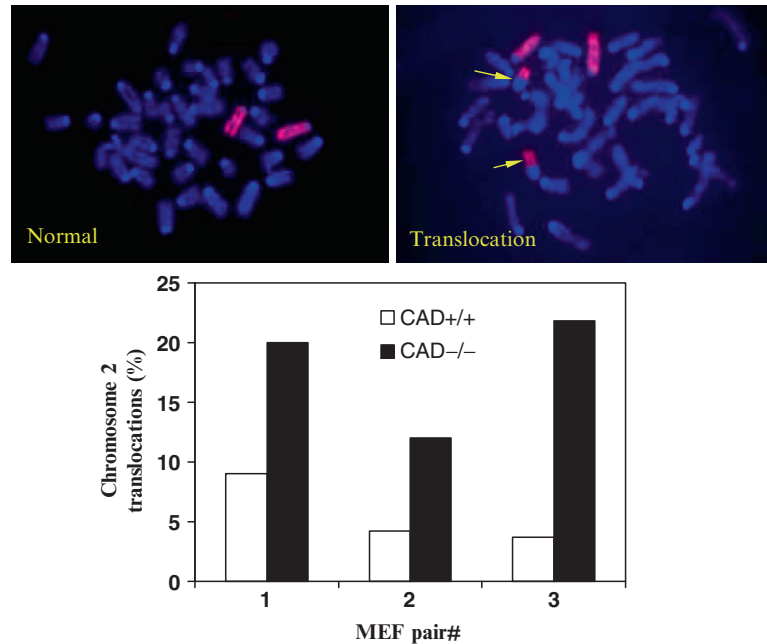


Fig. 1. Whole chromosome painting by FISH. MEF cells from CAD(+/+) and CAD(-/-) cells were irradiated with 4 Gy of γ -rays and then cultured for 12 days. Metaphase chromosome spreads were then prepared. Subsequently, chromosome painting was carried out for the chromosome spreads by use of fluorescently labeled whole chromosome 2 probes. The *top two panels* shows the fluorescent photomicrographs of typical spreads with normal (*left*) and translocated (*right*) chromosome 2. *Arrows* show the chromosomes that are involved in translocations. The *lower panel* shows elevated frequency of radiation-induced chromosome 2 translocations in embryonic fibroblast cells derived from CAD(-/-) mice. Results from three independent pairs of CAD(+/+) and CAD(-/-) mouse embryonic fibroblasts. For each condition, at least 100 metaphases were counted for each experimental point.

3.3. Detection of Aneuploid Cells by FACS Analysis

1. Cells were collected, washed with PBS + 1% FBS (*see Note 15*).
2. Cells are centrifuged and supernatant removed. Cold (4°C) 70% ethanol is added to the cell pellet drop by drop with gentle mixing to resuspend the cells. Fix the cells in cold ethanol for at least 3–6 h. At this point, cells can be left in the refrigerator for a couple of weeks.
3. Cells are centrifuged and stained with propidium iodide (10 µg/ml) and ribonuclease A (100 µg/ml) for at least 1 h but no more than 24–48 h.
4. Cells are then subjected to cell cycle analysis using FACS. A percentage of aneuploid cells was calculated with ModFit LT cell-cycle analysis software (8) (*see Notes 16–18*).

3.4. Micronucleus Assay

1. Cells were plated in the 6-well plates at about 20–30% confluence 24 h before treatment.
2. Treat cells with irradiation, TNF α or other genotoxic agents.
3. After treatment, cytochalasin B was added to the medium at the final concentration of 5 µg/ml and cultured for 48 h.
4. Then the medium was removed and the cells were rinsed with PBS and fixed by Carnoy fixative for 5 min. Then the cells were dried in the air.
5. Then the cells were immersed in the 2 \times SSC buffer with 0.1% NP 40 for 1 min.
6. After drying in the air, the cells were stained in acridine orange (AO) staining buffer for 2–5 min by gently shaking them (*see Note 19*).
7. Then the cells were washed with PBS and the micronuclei were scored under a fluorescent microscope. The criteria for identifying micronucleus is elaborated by Michael Fenech (9) (*see Notes 20–21*).

3.5. Immunodetection for 8-oxodG in Mouse Tissues

8-oxo-Deoxyguanosine (8-oxodG) is one of the major DNA lesions formed upon oxidative attack of DNA. It is an indicator of oxidative stress as well as a mutagenic adduct that has been associated with pathological states such as cancer and aging (10). The only available oxodG antibody is mouse monoclonal. In order to decrease the background staining due to the presence of endogenous mouse immunoglobins in the mouse tissue, the M.O.M.[™] immunodetection kit is used for the immunostaining. Below it is modified from the instruction of the kit.

1. The frozen tissue sections are fixed in ice-cold fixative (acetone:methanol = 1:1) for 10 min and then the slides are air-dried.
2. Wash sections in PBS for 4 min \times 3 times.

3. Incubate sections with 0.3% hydrogen peroxide in 0.3% normal horse serum in PBS for 10 min to quench endogenous peroxidase activity.
 4. Wash section 3 min \times 3 in PBS.
 5. Incubate sections for 1–4 h in working solution of M.O.M. mouse Ig blocking reagent.
 6. Wash slides for about 3 \times 3 min in PBS.
 7. Incubate slides for 5 min in working solution of M.O.M. diluents (*see* **Note 22**).
 8. Dilute 8-oxodG antibody in M.O.M. diluents to 1:1,000. Incubate section in diluted primary antibody for 30–60 min at room temperature or overnight at 4°C.
 9. Wash slides for about 3 \times 3 min in PBS.
 10. Apply working solution of M.O.M. biotinylated anti-mouse IgG reagent and incubate sections for 10 min (*see* **Note 23**).
 11. Wash slides for about 3 \times 3 min in PBS.
 12. Apply VECTASTAIN ABC reagent and incubate the sections for 5 min.
 13. Wash slides for about 3 \times 5 min in PBS.
 14. Prepare and apply peroxidase substrate solution according to substrate kit instructions and develop for 2–10 min with DAB or 2–15 min with VECTOR VIP (*see* **Notes 24–25**).
- See* **Fig. 2** for an example of the staining.

3.6. Soft Agar Assay for Cellular Transformation

One of the best in vitro indicators of a potential malignant growth is the ability of cells to grow in an anchorage-independent manner. Growth in semi-solid agar media is the most common assay (11).

1. Prepare the 0.5% agar bottom layer by mixing 1 volume of 5% agar (melted by microwave and cooled in 45°C water bath) with 9 volume of medium prewarmed to 45°C. Pipette 5 ml of the 0.5% agar/medium into each 60-mm dish and allow to solidify at room temperature (*see* **Note 26**).
2. Trypsinize the cells and prepare a serial dilutions of single cell suspension to 5×10^4 , 2×10^4 , 10^4 , 5×10^3 , 2×10^3 , 5×10^2 , and 2×10^2 cells/ml.
3. Briefly warm 1 ml of each dilution of cell suspension to 40°C and mix it with 2 ml of the warm 0.5% agar/medium to a final concentration of 0.33% agar in a 15-ml tube, then transfer the cells to the hardened 0.5% agar base layer (*see* **Note 27**).
4. Incubate cells at 37°C in a humidified 5–10% CO₂ environment. Feed cells twice a week by dropwise addition of the growth medium. Score for the presence and frequency of colonies after 2–3 weeks (*see* **Note 28**).

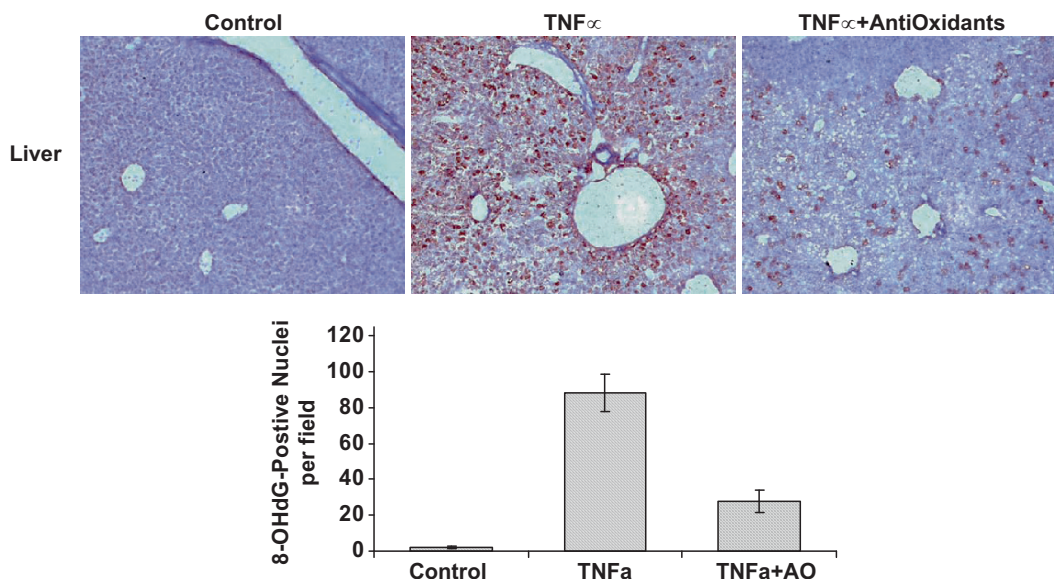


Fig. 2. Induction of 8-OHdG by $\text{TNF}\alpha$ in mouse liver tissue. Expression of $\text{TNF}\alpha$ in mice was achieved by hydrodynamic delivery of N1- $\text{TNF}\alpha$ expression plasmid. Four micrograms of DNA in a calculated volume were injected into each mouse over a period of 5 s. Mice were sacrificed 7 h after injection and tissues were removed and frozen in liquid nitrogen and subsequently stained with an antibody specific to 8-OHdG. The staining of liver tissue is shown in the *upper panel*. Quantification of the 8-OHdG-positive cells is in the *lower panel*.

- To confirm the tumorigenicity of the colonies that emerged in the soft agar assay, the colonies were picked, transferred into DMEM medium and expanded. Cells from each colony (about $3\text{--}5 \times 10^6$) were then injected subcutaneously into the right flanks of 6- to 8-week-old athymic nude mice. After inoculation, the incidence and growth of tumors were evaluated at least once a week for at least 16 weeks. Mice were sacrificed/dissected when tumor size reached 15 mm in diameter (*see Note 29*).

4. Notes

- When seeding cells to each well of a 12-well plate to determine the LD_{50} , seed more cells (up to 10^4) if the cells are small (such as HCT116), less cells ($2\text{--}5 \times 10^3$) if the cells are big such as fibroblasts.
- To compare cad gene amplification between different cell lines, the concentration of PALA/MTX for each cell line is determined by its LD_{50} . Use the same times of LD_{50} instead of the same concentration for each cell line.

3. Be very gentle when changing the medium, add the medium to the side of dishes in order not to flush the cell clumps and disperse them.
4. Only colonies with more than 50 cells are counted.
5. For slow growing cells such as fibroblasts, only the floating cells are collected for hypotonic incubation and fixation, every 3–5 h. Combine 2–3 harvests together. The cells in metaphase are round and not attached to the plate. Therefore we can concentrate these cells in this way.
6. Incubation in the hypotonic solution should not exceed 15 min otherwise the chromosome will be fussy; When resuspending the cells after spinning them down, suck out all but 200 μ l supernatant and resuspend the cells in this 200 leftover, then resuspend them to the final large volume.
7. Cells can be stored at 4°C for a couple of days at **step 4**. Cells can be stored in methanol at –20°C for longer time if necessary.
8. Dropping cell suspension onto slides is done in a blowing hood to accelerate drying for good spreading. Slides should be tilted and the height to drop is about 20–30 cm. Slides should be cleaned by detergent and water followed by ethanol and dried in the air before use.
9. Careful chromosome slide preparation is the first essential step of a successful hybridization. Usually prepare slides 1 day prior to hybridization. For a long-term storage, keep slides in a desiccator at –20°C. To use freshly made slides, incubate slides in 90°C oven for 10 min, followed by a 60-min incubation in 2 \times SSC at 37°C and dehydration in 70, 90, and 100% ethanol for 2 min each.
10. Slide pretreatment procedure may not be necessary if it is very clean. Enzymatic treatments reduce the background by digesting RNA and change the accessibility of the chromosome DNA by removing the cytoplasmic proteins.
11. For experiment of one slide, place the Coplin jar containing 70% formamide denaturation solution in the 70°C water bath approximately 30 min prior to use to bring the denaturation solution to 70°C. For every extra slide, increase the temperature setting 0.5°C to maintain the denaturation temperature at 70°C. Immerse no more than four slides in the solution simultaneously.
12. Start the probe denaturation during pretreatment and denaturation of chromosome slides. Time the procedure so that it is completed approximately the same time as the slide denaturation.
13. To prevent photo bleaching, handle all reagents and slides containing fluorochromes in reduced light.

14. Place the six Coplin jars containing two jars each of the 50% formamide in 2× SSC, 2× SSC, and PN buffer in the 42°C water bath approximately 30 min prior to use to bring the washing solutions to 42°C.
15. A normal diploid control should be set for each analysis. The best normal control is peripheral blood lymphocytes or bone marrow cells.
16. When comparing different samples, in order for the analysis to be accurate, equal amount of PI staining buffer should be added to stain equal amount of cells of different samples.
17. Cells should be analyzed within 24 h after staining for the best result.
18. The analysis should be performed by an experienced flow cytometry expert using the ModFit LT cell-cycle analysis software. Since researchers are generally not familiar with this software, it is important to have someone with experience to analyze the data.
19. The AO solution mixed with heptan form two layers. Take the lower portion containing AO to stain the cells.
20. If the nuclei look fussy under microscope when dry, 0.5 ml PBS can be added to the well and the image will become clear.
21. For the criteria of identifying MN or other nuclear abnormalities reflecting DNA damages such as the bridge reference can be seen in ?, *see* (9).
22. There are two tricks in decreasing the background caused by endogenous mouse Ig: (1) Permeabilize the tissue with 0.2% Triton X100 in PBS for 20 min at 4°C so that the endogenous Ig can be washed away in the following wash steps; (2) Use acetone instead of paraformaldehyde for fixation because paraformaldehyde can cross link proteins and make it hard to wash the endogenous Ig away.
23. Not all background present in a tissue section will be caused by endogenous mouse IgG. Appropriate negative control sections should be run in parallel, to rule out other possible causes of background.
24. Development times may differ depending upon the level of antigen, the intensity of the stain that is required or the substrate used.
25. During the staining procedure, do not allow the section to dry out. If necessary, use a humidified chamber for incubations.
26. The 0.5% agar/medium and the melted 5% agar should be kept in 45°C water baths during the experiment. They quickly solidify at room temperature.
27. In **step 3**, mixing the cell suspension with the 0.5% agar/medium should be done promptly and thoroughly before it solidifies.

28. It usually takes about 3 weeks for the colonies to form. Those with >50 cells are counted as transformed colonies.
29. The limitations of this assay are reflected in the observations that some normal cells do grow in suspension, and that many human tumor cells fail to grow in suspension (11). Therefore, the transformation property of cells forming soft agar colonies should be confirmed by tumorigenesis assay by inoculating cells into nude mice and observing tumor formation. Focus-formation assay can also be used to test cells that do not form colonies in soft agar medium.

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