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Detection of nucleotide disbalance in cells undergoing oncogene-induced senescence

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Abstract

DNA damage response has been characterized as an important mediator of senescence phenotypes induced by activated oncogenes in normal human cells. Depletion of intracellular deoxyribonucleotide pools has been recently recognized as one of the major causes for DNA damage in these cells. Cells undergoing oncogene-induced senescence display decreased expression of several rate-limiting enzymes involved in the biosynthesis of deoxyribonucleotides, including thymidylate synthase (TS) and ribonucleotide reductase (RR). Individual depletion of these enzymes leads to premature senescence. Reciprocally, ectopic expression of TS and RR or addition of deoxyribonucleosides resulted in suppression of senescence phenotypes in normal or tumor cells caused by overexpression of activated HRAS or depletion of C-MYC, respectively. Therefore, in the current chapter, we will describe the methodology for the quantitative measurement of nucleotide pools in senescent cells.

Introduction

Oncogene-induced senescence (OIS) in normal cells is characterized by a set of intracellular processes ultimately resulting in suppression of proliferation of pre-malignant cells (1–3). Due to this feature, OIS is considered a major impediment to oncogenic transformation (4). Compelling data demonstrates that DNA damage response is the major factor contributing to OIS (5–7). For a long time, oncogene-induced oxidative stress and hyper-replication of genomic DNA (multiple firing of the same origin of replication) were considered the two major causes for DNA damage in oncogene-expressing senescent cells. For instance, it has been demonstrated that generation of reactive oxygen species (ROS) via HRAS^{G12V}-dependent induction of NADPH oxidase 4 (NOX4) is an important mediator of HRAS^{G12V}-induced senescence (8). On the other hand, ectopic expression of activated HRAS in human fibroblasts caused multiple firing of the same replication origin of genomic DNA, which resulted in DNA replication fork-stalling, generation of single and double-stranded DNA breaks and ultimately, activation of the DNA damage response (7).

However, since then experimental evidence has been accumulated suggesting that suppression of nucleotide biosynthesis represents another major cause of DNA damage and OIS in normal and transformed cells. First, it has been demonstrated that upregulation of

treatment of certain types of tumors such as melanomas. Approximately 30 to 50% of melanomas originate from benign melanocytic nevi, aggregates of senescent melanocytes most commonly expressing mutant BRAF^{V600E} (~60%) or NRAS^{Q61R} (~20%) (19, 20). Therefore, melanoma must develop mechanisms overcoming OIS and upregulation of nucleotide metabolism appears to be one of the most likely candidates.

In this chapter, we will describe a comprehensive methodology for measuring the intracellular amounts of ribo- and deoxyribonucleotides in control cells and cells undergoing HRAS^{G12V}-induced senescence; however, the same methodology could be applied to any senescent cells.

2. Materials

2.1 Cells and growth media

1. WI38 cells (normal human lung fibroblasts).
2. HEK293T cells (human embryonal kidney cells transformed with the SV40 large T antigen).
3. DMEM supplemented with antibiotic-antimycotic and 10% fetal bovine serum).

2.2. Reagents and materials for lentiviral gene transfer

1. SuperFect[®] transfection reagent
2. 0.45 µm syringe filters

2.3 Plasmids

1. FG12 lentiviral vector.
2. FG12-HRAS^{G12V} was generated by re-cloning HRAS^{G12V} cDNA from pcDNA3-H-Ras_V12 plasmid into FG12 lentiviral vector.
3. pCMV-VSV-G
4. pCMV delta R8.2.

2.4 Reagents for Nucleotide Extraction (see Note 1

1. Perchloric Acid: 70% reagent grade. Dilute with HPLC-grade water to 0.8 N.
2. Potassium Hydroxide: Dissolve potassium hydroxide in HPLC-grade water to make 10 N and 1 N stocks.

¹HPLC grade water is more reliably free of UV-absorbing impurities than lab deionized or Millipore-purified water. All flasks and beakers used to prepare and store buffers should be used for HPLC buffers only. Immediately after use, rinse all equipment thoroughly with lab deionized water followed by a final rinse with HPLC grade water, then air dried in a clean area. If the containers need to be washed with soap, afterward rinse thoroughly with lab deionized water, and then use a final rinse with HPLC-grade water.

2.5. Reagents for Boronate Chromatography

1. Ammonium bicarbonate buffer, 1 M, pH 8.9: dissolve ammonium bicarbonate in HPLC-grade water, bring the pH to 8.9. Use this stock to make 0.05 M ammonium bicarbonate, pH 8.9.
2. Magnesium chloride, 0.3 M: dissolve magnesium chloride in HPLC-grade water. Use an aliquot of this stock to make a stock of 0.015 M magnesium chloride.
3. Borate buffer, pH 8.9: dissolve borate buffer in HPLC-grade water and pH to 8.9.
4. Hydrate Affi-Gel Boronate Affinity Chromatography gel in 0.05 M ammonium bicarbonate, pH 8.9, for at least 15 min with several changes of buffer. Pack the hydrated gel suspension into a 1 cc tuberculin syringe fitted with a frit (to retain the gel), and pack to a height of approximately 0.9 mL. Connect a 10 mL syringe onto the top of the Affi-Gel column as a buffer reservoir. Equilibrate the column with at least 10 mL of 0.05 M ammonium bicarbonate buffer, pH 8.9, with 0.015 M magnesium chloride. Run at least 10 mL of 0.1 M borate buffer, pH 8.9, through the column. If not using immediately, store the column at 4°C in 0.1 M borate buffer, and wrap tightly with parafilm.

2.6 HPLC buffers and solvents

1. HPLC-grade methanol
2. HPLC buffers should be made with HPLC-grade ammonium phosphate and dissolved in HPLC-grade water.

0.005 M ammonium phosphate, brought to a pH 2.8 with concentrated phosphoric acid

0.75 M ammonium phosphate, brought to a pH of 3.6 with concentrated phosphoric acid

After preparing the buffers, they should be filtered through a glass or Teflon filter-degasser apparatus with a 0.45 µm filter under vacuum (the apparatus has an arm to attach to the vacuum arm on a lab water faucet). This type of filter apparatus is available from several suppliers. We use a teflon filter-degasser with a cap that screws onto a standard 4 L solvent bottle. These bottles can then be used for buffer storage, or the HPLC buffer line can be placed directly into the bottle. If you choose to transfer the buffer to another glass container, pour the buffer in carefully to limit aeration (see Note 1).
3. EDTA: Prepare 0.1 M stock of EDTA in HPLC-grade water. Filter through a 0.22 µm filter housing.

2.7 Preparation of NTP standards

1. Make individual stocks of high purity rNTPs (CTP, UTP, ATP and GTP) and dNTPs (dCTP, dTTP, dATP and dGTP) separately in HPLC-grade water. Check UV absorbance for each nucleotide to ensure you have the correct concentration. (see Note 2).

2. Make a 10 μ M stock solution for the four rNTPs, and a 10 μ M stock for the four dNTPs.

2.8 HPLC Equipment

1. Gradient HPLC system equipped to run at least four different solvents with a 2 mL injection loop.
2. UV detector for gradient HPLC (photodiode array detector)
3. Partisil 10 SAX analytical column (quaternary ammonium ion), 25 cm $\times$ 4.6 mm
4. Anion exchange guard column and housing
5. Silica pack and housing

3. Methods

3.1 Generation of human fibroblasts undergoing oncogene-induced senescence

1. Day 1. Plate

3.2 Extraction of nucleotides from cells

Perform all procedures with ice-cold reagents, and keep samples on ice during the entire process.

1. Harvest cells from culture by trypsinization, then dilute cells in complete growth medium (with serum) to stop trypsinization. Pipet cells up and down a few times to obtain a single cell suspension. Count an aliquot of cells (Coulter electronic particle counter or hemocytometer) for calculation of total number of cells extracted. Keep cells for extraction on ice. (*see* Note 3).
2. Centrifuge cells at 250 $\times$ g, 4°C, for 5 min (~1200 rpm on a centrifuge of ~16 cm radius).
3. Aspirate supernatant from the cell pellet. Remove as much of the cell growth medium in the supernatant as possible and discard. Keep cell pellet on ice.
4. Place conical tube with cell pellet on vortex, then rapidly add ice-cold 0.5 mL HPLC-grade water. Use an Eppendorf pipetter to expel water onto the cell pellet while vortexing to help resuspend cells. Vortex well to resuspend cell pellet.
5. While continuing to vortex cells, rapidly add 0.5 mL of ice-cold 0.8 N perchloric acid. Continue to vortex for a few seconds to mix contents. Return cell extract to ice for 5 min.
6. Centrifuge cell extract at 250 $\times$ g, 4°C, for 5 min.

²We check the purity of each freshly made nucleotide standard by running it on HPLC against standards of known concentration.

³Cells for extraction should be centrifuged in a conical (not a round-bottom) tube so that the cells will pellet well to the bottom, and there will be better recovery of the supernatant during extraction. A 15 to 50 mL plastic conical tube works well, depending on the number of cells to be extracted. For most cultured tumor cell lines, dNTPs can be accurately measured in 10 to 20 million cells.

7. Carefully remove supernatant from pellet and place in a fresh centrifuge tube. Keep supernatant at 4°C. Discard pellet.
8. Neutralize supernatant with ~40 µL 10 N KOH while vortexing. Measure approximate pH by pipetting 1 µL of supernatant onto pH paper. If the pH is less than 6, add aliquots of 1 µL each of 1 N KOH to bring final pH to ~ 7 on pH paper.
9. Centrifuge neutralized extract at 400 × g, 4°C, for 5 min. Remove supernatant to fresh tube. Freeze at –20°C until analysis. The extracts should be quite stable when stored frozen at neutral pH. Discard precipitate.

3.3 Separation of dNTPs from rNTPs by boronate chromatography

1. Bring the boronate columns to room temperature.
2. Equilibrate the boronate columns with at least 10 mL of 0.05 M ammonium bicarbonate buffer, pH 8.9.
3. To 0.9 mL of cell extract, add 50 µL of 1 M ammonium bicarbonate, pH 8.9, and 50 µL of 0.3 M magnesium chloride. Vortex well.
4. Load 0.2 mL of the buffered cell extract to the top of a boronate column; discard the eluate. Load the remaining 0.8 mL onto the top of the boronate column and collect the eluate. When the sample has fully penetrated into the column matrix, add 1 mL of 0.05 M ammonium bicarbonate, pH 8.9, with 0.015 M magnesium chloride and collect the eluate. *The 1.8 mL eluate contains the dNTPs; place on ice immediately and save for HPLC analysis.*
5. To remove the rNTPs from the boronate column, elute with at least 10 mL of 0.1 M borate buffer, pH 8.9.
6. Just prior to HPLC analysis, bring the pH of the dNTP eluate to ~2 to 3 with phosphoric acid. Keep the acidified eluate on ice, and analyze promptly. (see Note 4).

3.4 Separation of nucleotides by HPLC

1. All solvents and buffers should be HPLC grade. The HPLC column and buffers are run at room temperature. All samples should be stored on ice until they are injected onto the HPLC column.
2. Column setup: the SAX guard column should be inserted in its housing and attached to the SAX analytical column. The silica pack is inserted upstream from the guard and analytical column, in the tubing that carries the mixed buffer solution, so that the mobile phase will be saturated with silica prior to running on the analytical column.

⁴Add the magnesium chloride to the ammonium bicarbonate buffer just before using it. Precipitates will form with time in this buffer combination and impeded the flow through the boronate column and HPLC. Flow through the boronate columns is slow. You may wish to prepare several boronate columns so that you can run several samples simultaneously. The boronate chromatography procedure will remove 99.9% of the rNTPs, with >95% recovery of the dNTPs. Each column can process dozens of samples. When the column needs to be replaced, you will note an increase in the amount of rNTPs in the dNTP eluate during the HPLC analysis.

3. The SAX column should be stored in 100% methanol. To prepare the column for running NTPs, the mobile phase must first be changed from methanol to water. Use a linear gradient from 100% methanol to 100% water over 30 min at 1 mL/min. Set the UV detector to the desired wavelength for your samples.
4. Continue to run 100% water through the column for another 30 min at 1 mL/min. Increase flow rate to 2 mL/min.
5. Change the mobile phase to 50% 0.005 M, pH 2.8, and 50% 0.75 M, pH 3.6, ammonium phosphate buffer and equilibrate the column for 15 min.
6. Inject 2 mL of 0.1 M EDTA. Run a linear gradient from 50% 0.75 M buffer to 100% 0.75 M buffer over 15 min, then continue to elute the EDTA with 100% 0.75 M buffer for another 15 min.
7. Run a linear gradient from 100% 0.75 M buffer to 20% 0.75 M and 80% 0.005 M buffer over 10 min. Equilibrate column for at least 30 min.
8. Inject 0.5 nmol dNTP standards and 0.25 nmol rNTP standards and elute with a linear gradient of 80% 0.005 M and 20% 0.75 M buffer to 20% 0.005 M and 80% 0.75 M buffer over 30 min, then continue to elute at this buffer concentration for another 5 min. The nucleoside triphosphates will elute between ~18 and 30 min. (see Note 5).
9. Run a linear gradient from 20% 0.005 M and 80% 0.75 M buffer to 80% 0.005 M and 20% 0.75 M over 10 min to return the column to the starting buffer concentration.
10. Allow the column to equilibrate for at least 30 min in between runs.
11. For sample analysis, centrifuge samples at $400 \times g$, 4°C, for 5 min to remove any precipitate. Carefully pipet the amount of sample needed for analysis. Inject samples using the same conditions as in **steps 8 and 9**.
12. After several runs, the baseline will begin to increase due to impurities in the buffers. Run 2 mL of EDTA using the gradient conditions in **steps 6 and 7** above to decrease the baseline absorbance. Make sure to equilibrate the column well after EDTA until the absorbance has stably returned to baseline.
13. To store the column, change the mobile phase from buffer to water using a linear gradient from 80% 0.005 M and 20% 0.75 M to 100% water over 15 min at 2 mL/min. Equilibrate column in 100% water for at least 30 min, 2 mL/min. Change the mobile phase from 100% water to 100% methanol over 30 min at 1 mL/min. Equilibrate for at least 30 min in 100% methanol, 1 mL/min, before shutting off the machine. Be careful not to change the mobile phase abruptly

⁵If any of the dNTPs coelute with the rNTPs or do not achieve baseline resolution, try altering the pH of the 0.75 M ammonium phosphate buffer in increments of 0.1 pH units. Decreasing the pH will increase the area between CTP and UTP, whereas increasing the pH will increase the area between ATP and GTP at the expense of decreasing the CTP and UTP separation. In most cases, there are no detectable rNTPs when the boronate-purified dNTP eluate is analyzed by HPLC. However, depending on the number of cells analyzed and the condition of the column, there may be slight contamination of the dNTP fraction with rNTPs. Thus it is necessary to make sure that you can separate the rNTPs from the dNTPs by HPLC analysis.

from methanol to ammonium phosphate buffer, as the buffer will precipitate and clog the HPLC.

14. Integrate the area under the curve for the NTP standards, and then calculate the integrator units/nmol based on the known amount of each standard injected. The amount of dNTPs in each sample is then calculated based on the integrator units/nmol of the appropriate standard dNTP.

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