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Cellular Identification and Quantification of Senescence-Associated β -Galactosidase Activity In Vivo

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Abstract

Senescence-associated β -galactosidase (hereafter SA- β -gal) staining has now been employed for more than 20 years to identify the presence of senescent cells (Dimri et al., Proc Natl Acad Sci U S A 92:9363–9367, 1995). These cells, characterized by a permanent cell-cycle arrest (Hayflick and Moorhead, Exp Cell Res 25:585–621, 1961) and the production of a distinct secretory phenotype of cytokines, chemokines, and proteases (Coppe et al., PLoS Biol 6:2853–2868, 2008), have received much attention in recent years for their impacts on diverse biological processes. Here we describe a method to identify and quantify the specific cells that become senescent in vivo using transmission electron microscopy after SA- β -gal staining that can be used in countless scenarios.

Keywords

Cellular senescence; Transmission electron microscopy; Senescence-associated β -galactosidase

1 Introduction

Cellular senescence, a state of stable growth arrest [2] characterized by the acquisition of a distinctive secretome [3], has been linked to various biological processes. From a beneficial perspective, senescent cells limit the capacity for pretumorous lesions to proliferate, direct embryogenesis [4, 5], and mediate tissue remodeling [6, 7] and regeneration [8]. However, senescent cells also accumulate with age and at sites of pathology, where they have been shown to drive tissue deterioration [9, 10] and promote various age-related diseases, including osteoarthritis [11] and atherosclerosis [12]. As such, identification of senescent cells in vivo has become a major scientific objective. Although a variety of biomarkers have been used for this purpose, including expression of the cyclin-dependent kinase inhibitor p16^{Ink4a}, the single most common approach is the enzymatic detection method for senescence-associated β -galactosidase (SA- β -gal). Briefly, on exposure to a chromogenic substrate (X-Gal; 5-bromo-4-chloro-3-indolyl- β D-galactopyranoside; see Fig. 1), a lysosomal hydrolase active in SNCs at pH 6.0 produces a blue precipitate, which is usually detected by light microscopy [1]. The ease with which this assay is performed, as well as its

compatibility with fresh or frozen cryosectioned tissue or cells, has led to its widespread implementation.

Here, we elaborate on a refinement of SA- β -gal staining that we call GAL-EM: ultrastructural analysis by transmission electron microscopy (TEM) of SA- β -gal-stained tissue for SNC identification, quantitation, and localization with respect to tissue features. Briefly, we serendipitously discovered that the X-Gal precipitate produced by SA- β -gal activity appears as distinctive cuboidal, electron-dense needles when examined by TEM [9, 12]. It was also readily apparent that tissue architecture and cellular morphology following immersion staining of tissue fragments generally permits cell-type identification.

Importantly, we validated two key aspects of this finding. First, the crystals we observe are definitively precipitates of X-gal. X-ray elemental analysis confirmed the presence of chlorine and bromine heteroatoms in crystals but not adjacent cellular material (Fig. 1). Furthermore, incubation of tissue in X-gal staining solution lacking only X-gal did not produce these crystals. Second, the incidence of crystal-positive cells increases with age in mouse inguinal white adipose tissue, kidney, and pericardium and is reduced to youthful levels with transgene mediated senescent cell clearance using the transgenic INK-ATTAC mouse model system [9].

2 Materials

For SA- β -gal staining, we typically use the reagents provided by Cell Signaling Technology catalog #9860. All solutions are prepared using ultrapure water (prepared by purifying distilled water to attain a sensitivity of 18.2 M Ω -cm at 25 °C). Following SA- β -gal staining, samples are transferred to freshly prepared Trump's fixative for processing for transmission electron microscopy (TEM).

2.1 Senescence-Associated β -Galactosidase (SA- β -Gal) Staining

1. 1 $\times$ phosphate buffered saline (PBS): 1.7 mM KH₂PO₄, 5 mM Na₂HPO₄, 150 mM NaCl (pH 7.4).
2. 10 $\times$ fixative solution: 20% formaldehyde, 2% glutaraldehyde in 10 $\times$ PBS.
3. 10 $\times$ staining solution: 400 mM citric acid–sodium phosphate (pH 6.0), 1.5 M NaCl, 20 mM MgCl₂.
4. 100 $\times$ staining supplement A: 500 mM potassium ferrocyanide.
5. 100 $\times$ staining supplement B: 500 mM potassium ferricyanide.
6. *N-N*-dimethylformamide (DMF).
7. X-gal: 5-bromo-4-chloro-3-indolyl- β D-galactopyranoside powder. Dissolve 20 mg X-gal in 1 mL DMF to prepare a 20 $\times$ stock solution. This stock solution can be stored at –20 °C for up to 1 month in a light resistant tube. Make sure tubes are not polystyrene, use only polypropylene or glass.
8. Freshly prepare staining solution prior to use in polypropylene tubes. Per 1 mL of staining solution, you will require:

- a. 930 μ L 1 $\times$ staining solution
 - b. 10 μ L of 100 $\times$ staining supplement A.
 - c. 10 μ L of 100 $\times$ staining supplement B.
 - d. 50 μ L 20 mg/mL X-gal in DMF.
9. 37 °C incubator (not water jacketed).
 10. 48-well flat bottom plates.
 11. pH meter.

2.2 Transmission Electron Microscopy (TEM)

1. Cacodylic acid.
2. 16% paraformaldehyde.
3. 8% glutaraldehyde solution.
4. Magnesium chloride (MgCl_2).
5. Nanopure H_2O .
6. Sodium hydroxide (NaOH).
7. Trump's fixative (100 mM cacodylic acid; 4% paraformaldehyde; 1% glutaraldehyde; 2 mM MgCl_2). To make 40 mL, add 0.56 g cacodylic acid to 20 mL of nanopure H_2O . Increase pH to 7.2 with NaOH . Add 10 mL of 16% paraformaldehyde solution, 5 mL of 8% glutaraldehyde, and 80 μ L of 1 M MgCl_2 . Bring total volume up to 40 mL total with nanopure H_2O .
8. Jeol 1400+ electron microscope.

3 Methods

3.1 Senescence-Associated β -Galactosidase (SA- β -Gal) Staining

1. Dilute 10 $\times$ fixative and 10 $\times$ staining solution with purified water to make 1 $\times$ solutions. You will need ~500 μ L per tissue you plan to stain.
2. Dissolve 20 mg X-gal in 1 mL DMF to prepare a 20 $\times$ stock solution. This stock solution can be stored at -20°C for up to 1 month in a light resistant tube. Make sure tubes are not polystyrene, use only polypropylene or glass.
3. Dissect out all tissues of interest and place in ice-cold PBS in 48-well plate on ice until complete. Proceed quickly to minimize time on ice. Once all tissues are isolated, proceed with all to fixation. Perfusion may be necessary for some tissue samples (*see* Note 1).

¹In some situations, perfusion of the animal may be required to clear the blood from tissues prior to SA- β -gal staining. In these scenarios, perfusion with the fixative should proceed immediately after clearance to preserve tissue structure and viability. Animals are anesthetized through a ketamine/xylazine injection and transcardially perfused with ice-cold PBS until no further blood leaves the heart. If performed appropriately, the kidneys and liver should change color from red to light tan. Then, 10 mL of fixative is perfused through the animal at a rate of 3.33 mL/min for 3 min. Excess fixative is removed through a 1-min wash with chilled PBS at the same

4. Transfer tissue to fixative and fix at room temperature. Suggested fixation time can vary depending on the tissue, but in general a 20-min fixation is suitable. Some tissue may require different fixation methods (*see* Note 2).
5. While in fixative, freshly prepare the staining solution in polypropylene as described in Subheading 2.1, **item 7**.
6. After fixation, rinse tissue twice in PBS.
7. Transfer tissue to staining solution and incubate at 37 °C in the dark. The duration of staining varies by tissue and will need to be monitored for the development of blue color. We have found that 12 h works best for adipose tissue (Fig. 2) and 48 h consistently stains heart (Fig. 3) and kidney (Fig. 4) tissue of aged animals [9]. In the atherosclerosis disease model [12], 12 h works best for plaque containing regions (Fig. 5). *See* Note 3 for an experimental method to determine optimal staining duration. Tissues can be moved to 4 °C to retard further color development.

3.2 Transmission Electron Microscopy (TEM)

1. Make fresh Trump's fixative as described above prior to each experiment.
2. Following SA- β -Gal staining, transfer tissue to Trump's fixative at 4 °C for 12 h.
3. Routinely process these pieces for OsO₄/lead staining to detect X-Gal crystals by transmission electron microscopy (TEM) as described [13].
4. Acquire images and quantify performed on a Jeol 1400+ electron microscope (Jeol) with 80 kV acceleration voltage. To measure the percentage of X-Gal-crystal-containing cells, screen at least 5 grids per tissue for cells that are X-gal-positive cells at 3000–15,000 $\times$ magnification depending on the tissue type. Cells with one or more crystals and the total number of cells should be counted. Representative crystal containing cells can be found in Fig. 2.

rate. For brain tissue, subregions are placed directly into β -gal staining solution after dissection, as the traditional fixation step has occurred via perfusion.

²Although the traditional SA- β -gal kits utilize a 20% formaldehyde/2% glutaraldehyde fixation method, certain tissues are prone to tearing and loss of microstructure with this fixative. For brain tissue, we have found that tissue architecture is significantly improved using the perfusion technique described above (*see* Note 1) through perfusing the animal with chilled 4% PFA. Perfusion rates and volumes all remain identical as described above.

³While some tissues easily present overt differences in SA- β -gal staining, other tissues require optimization. Staining durations that are too short can produce false negatives, while extended staining periods can result in false positives. To optimize staining, a positive and negative control sample is required. Samples should be collected from each animal, and prepared following the traditional staining steps (*see* Subheading 3.1). Samples from each should be placed into staining solution and incubated at 37 °C. After 6 h, samples should be removed and checked for a shift in overt color. If the positive control sample is overtly blue and the negative is not, samples can be shifted to Trump's fixative for EM processing. If not, return the samples to the staining solution and recheck them every 6 h until they are. If the samples do not overtly show a difference, or they present as overtly blue at the same time, the next step is to assess sample specificity using sections prepared for light microscopy. Place samples in staining solution until for 6 h, then fix in 4% PFA at 4 °C for 48 h. Process tissues for paraffin embedding using routine methods and section samples 7 μ m thick. Make sure to collect samples deep enough into tissue to prevent false positive staining, as edges and cut surfaces tend to stain positively. Perform analysis by light microscopy by looking for cell-specific staining in the positive control sample and a lack of the same staining in the negative sample. If staining is absent, increase the incubation period by 6 h and repeat. From our experience, if a tissue is going to stain positively, it will do so prior to 72 h of incubation in the staining solution.

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Fig. 1. Validation of SA- β -gal crystals by elemental analysis. **(a)** Chemical structure of X-gal. **(b)** Senescent proximal tubule cell following SA- β -gal staining and TEM on kidney tissue containing a crystal (box). **(c)** Elemental analysis of neighboring area (left, black arrow in **b**) and crystal (right, red arrow in **b**). The crystal has a profound increase in Br signal due to the X-gal precipitate

Fig. 2.

Representative image of X-gal crystals in white adipose tissue following SA- β -gal staining and TEM. *A* adipocyte, *C* capillary. Arrows mark endothelial cells. Scale bars: 2 μ m and 200 nm (inset; reproduced from ref. [9] with permission from Nature)

Fig. 3. Representative image of X-gal crystals in pericardial tissue following SA- β -gal staining and TEM. Electron micrograph of X-gal positive cells in the pericardium (asterisk denotes cilia). Scale bars: 2 μ m and 200 nm (inset; reproduced from ref. [9] with permission from Nature)

Fig. 4.

Representative image of X-gal crystals in renal tubule cell following SA- β -gal staining and TEM. A renal epithelial cell with a defined brush border membrane (arrowheads) is shown. Scale bars: 5 μ m and 200 nm (inset; reproduced from ref. [9] with permission from Nature)

Fig. 5.

Representative image of X-gal crystals in atherosclerotic lesions following SA-β-gal staining and TEM. Electron microscopy shows three types of senescent cells in plaques of *Ldlr*^{-/-} mice on a high-fat diet for 88 days. Cell outlines are traced by dashed lines. Scale bars: 2 μm and 500 nm (inset; reproduced from ref. [13] with permission from Science)