CHAPTER TEN

NF-κB as a Determinant of Distinct Cell Death Pathways

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Contents

Abstract

The NF-κB signaling system has important and distinct roles in determining cell fate decisions, such as cell proliferation and cell death. Specifically, recent evidence indicates that NF-B regulates several types of programmed cell death, such as apoptosis, necroptosis, necrosis, as well as cellular senescence, but its precise role in these is not fully understood. Distinguishing these cell fates experimentally is therefore important, and several techniques are available to researchers. We summarize experimental strategies and protocols that reveal changes in nuclear morphology and cell shrinkage, exposure of phosphatidylserine, compromised membrane integrity, DNA fragmentation, and altered

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mitochondrial membrane potential. Together, these may discriminate distinct cell death pathways and lead to a better understanding of the underlying regulatory mechanisms.

1. Introduction

Starting with the genetics of *Clostridium elegans*, the molecular mechanisms underlying developmentally programmed cell death have been characterized over the past 20 years or so. In humans, negative selection of T cells by means of the death receptor Fas provided a similar starting point. The speed and success of these studies may have given the impression that this type of cell death—apoptosis—is the most important, the most common, or even the only one that fits the description of programmed cell death. However, other forms of programmed cell death, such as necrosis and cellular senescence, have regained prominence in a number of physiologic and pathologic settings.

Our understanding of NF-κB's role in cell death has similarly evolved. Although this transcription factor was first identified as a B-cell developmental regulator and was then associated more broadly with inflammation and immune responses, knockout mice deficient for the ubiquitous family member RelA were found to be embryonic lethal because of massive apoptotic cell death in fetal liver hepatocytes (Beg *et al.*, 1995). That finding established NF-κB as a major anti-apoptotic or "pro-survival" regulator. In recent years, NF-κB, which neither exists in *C. elegans* nor plays a major role in Fas-induced apoptosis, has been increasingly implicated in regulating a variety of cell death pathways, and not always as a pro-survival transcription factor (Fig. 10.1).

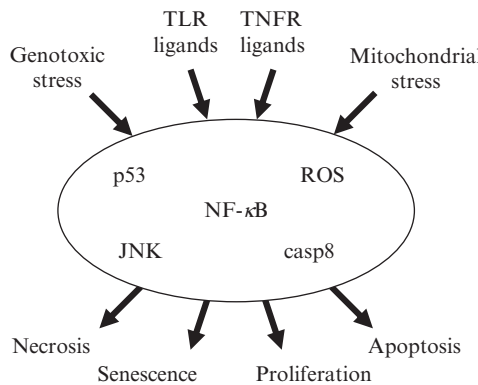

Figure 10.1 Signaling networks involving NF-κB that control cell fate. Diverse signals impinging on cells activate different combinations of signal transducers, only some of which are shown in the schematic. The network of interactions produces different cellular responses. NF-κB has been implicated in the control of cell proliferation, apoptosis, necrosis, and senescence.

Genetic ablation of NF-κB/RelA led to embryonic lethality because of massive apoptosis in the embryonic liver brought about by embryonic tumor necrosis factor (TNF) signaling (Alcamo *et al.*, 2001). TNF was previously known to kill cultured cells sensitized by treatment with the ribosome inhibitor cycloheximide—the new results suggested that NF-κB was responsible for this *de novo* gene expression (Beg and Baltimore, 1996). Subsequent screens for NF-κB target genes that act as anti-apoptotic regulators produced several candidates, including Bcl2 family member Bfl/A1, FLIP, A20, cIAPs, ferritin heavy chain (FHC), and Gadd45ß, (Papa *et al.*, 2004), but it remains unclear which are the relevant targets in specific physiologic contexts. However, blockade of NF-κB activity, either genetically by Iκ B super-repressor expression or RNAi, or pharmacologically, renders many cell types sensitive to apoptosis in response to diverse stimuli. Cancer cells, in particular, seem to lose their chemoresistance when elevated NF-κB activity is reduced by either of these strategies.

However, in other experimental scenarios, NF-κB was shown to play a pro-death role. The first such report showed that Sindbis virus caused cell death in an NF-κB–dependent manner (Lin *et al.*, 1998). More recently, UV irradiation was shown to signal through PKCδ, whose expression is NF-κB–dependent, toward a death pathway involving JNK (Liu *et al.*, 2006). These studies identified homeostatic NF-κB activity, and not stimulus-induced NF-κB activity, as being responsible for determining cell death sensitivity. Another study used a panel of IKK knockout cells and distinguished between apoptotic and necrotic cell death; whereas NF-κB–deficient cells were sensitive to apoptosis, chemical inhibition of caspases increased the amount of necrotic cell death (May and Madge, 2007). Furthermore, chronic NF-κB activity is also associated with cell senescence or aging (Adler *et al.*, 2007). Senescent cells are not dead, but they are unable to proliferate or exit G0, and physical manipulation (trypsinization) will often cause cell death.

Thus, NF-κB seems to be involved in a host of cell fate decisions that often fall within the cell death category. Genes known to be important regulators of these processes have been identified as NF-κB targets, including the anti-apoptotic regulators (mentioned earlier), anti-reactive oxygen species (ROS), regulators (ferritin heavy chain, FHC; superoxide dismutase, SOD; catalase), and cell cycle regulators (p21, cyclin D). The role of NF-κB is clearly cell type specific, as well as stimulus specific. TNF Receptor and Toll like Receptor superfamilies, as well as metabolic and genotoxic stress agents, engage a number of different signaling pathways. On the basis of recent publications, it seems that the role of NF-κB may, in part, be determined by the status and activity of the JNK pathway, as well as the p53 regulatory system (Gurova *et al.*, 2005; Ryan *et al.*, 2000). However, much more needs to be done to produce a predictive model. Therefore, distinguishing between the different types of cell death is an important component to understanding NF-κB's role in determining specific death pathways.

2. Methods

2.1. Electron microscopy

Both apoptotic and necrotic cell death can be determined by their unique physiologic hallmarks. One recognized characteristic of cells undergoing apoptosis includes change in nuclear morphology, such as chromatin condensation (pyknosis) followed by chromosomal DNA fragmentation (karyorrhexis). Cell shrinkage and the formation of apoptotic bodies that contain cellular material all occur before the loss of plasma membrane integrity (Kerr *et al.*, 1972). In contrast, the morphologic characteristics of necrotic cells do not display these criteria that define apoptosis. Necrotic cells show early plasma membrane rupture and the presence of cellular debris (Kroemer and Martin, 2005). Visualization by electron microscopy is a very clear way to distinguish between these two types of cell death.

Carefully suspend cells in fresh cell culture medium. Add an equal volume of Karnovsky's fixative (1.5% glutaraldehyde, 3% paraformaldehyde, 5% sucrose in 0.1 *M* cacodylate buffer, pH 7.4) and incubate at room temperature for 15 min. Wash cells three times in 0.1 *M* cacodylate buffer with slow speed centrifugation. Postfix in 1% OsO_4 in 0.1 *M* cacodylate buffer for 1 h at room temperature. Wash three times in cacodylate buffer followed by one wash in water. Carefully resuspend in 10% ethanol and stain *en bloc* in 1% uranyl acetate in 10% ethanol for 1 h in the dark. Dehydrate with a series of ethanol washes (25%, 50%, 75%, 95%), incubating 10 to 20 min each. Finally, wash two times in 100% ethanol. Dehydrate in a 1:1 mixture of propylene oxide and 100% ethanol for 20 to 30 min, followed by 100% propylene oxide. Treat samples 2 to 4 h each with increasing mixtures of resin (1:3, 1:1) and leave overnight in 3:1 resin. The next day, transfer samples into 100% resin for a few hours. Repeat twice with fresh 100% resin before finally transferring samples to embedding capsules with fresh resin. Polymerize overnight at 60 °C. Cut ultrathin sections with an ultramicrotome and contrast with 1% uranyl acetate and lead nitrate. Examine sections with a transmission electron microscope. (Protocol designed by K. Kudlicka.)

2.2. Membrane health

2.2.1. Distinguishing between apoptosis and necrosis

Lipids in biological membranes are asymmetrically distributed on either side of the bilayer as an active process, depending on the origin of lipid synthesis and enzymatic maintenance (Daleke, 2003). Cells undergoing apoptosis lose membrane asymmetry, and this has been used as a distinctive marker to discriminate apoptotic cells from other forms of cell death. Membrane integrity (i.e., the

exclusion of normally membrane-impermeable molecules) is maintained through most of the apoptotic process, whereas necrotic cells rapidly lose integrity of the plasma membrane (Golstein and Kroemer, 2007). These categorical differences can be useful in distinguishing between the two death processes. The caveats are that, ultimately, all dying cells lose membrane integrity, and as necrotic cells lose membrane integrity, they can appear to have lost asymmetry as probes gain access to the intracellular side. Specifically, Annexin V binding is not a feature restricted to apoptosis, because cells undergoing necrosis can also appear positive (Lecoeur *et al.*, 2001). Likewise, it has been shown that early apoptotic and early necrotic cells are $7AAD^{lo}$Annexin V^+PI^-, whereas both late apoptotic and necrotic cells are $7AAD^{hi}$Annexin V^+PI^+ (Lecoeur *et al.*, 2002). However, others have published the ability to distinguish between apoptotic and necrotic cells with the simultaneous staining of Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (Matteucci *et al.*, 1999). With these caveats in mind, cell death can be analyzed productively by taking advantage of these mechanistic distinctions.

2.2.2. Detection of extracellular phosphatidylserine

In a viable cell, phosphatidylserine is found on the cytosolic surface of the plasma membrane. Cells undergoing apoptosis lose membrane asymmetry, exposing phosphatidylserine on the extracellular surface (Daleke, 2003). The translocation of this phospholipid marks the cells for removal by phagocytosis through macrophages (Henson *et al.*, 2001).

To detect exposed phosphatidylserine by Annexin V binding (Vermes *et al.*, 1995), cells can be stained with phycoerythrin (PE)- or allophycocyanin (APC)-labeled Annexin V (Caltag Laboratories, Invitrogen, cat. no. ANNEXINV04, ANNEXIN05). Because Annexin V binds more specifically to negatively charged phosphatidylserine in the presence of calcium, staining should be performed in Annexin V binding buffer (140 m*M* NaCl, 2.5 m*M* $CaCl_2$, 10 m*M* HEPES). Wash 0.5 to 1 $\times$ 10^6 cultured cells with PBS by centrifuging at 1400 rpm for 5 min, resuspend in 100 μl of Annexin V diluted 1:150 in binding buffer, and incubate for 15 min in the dark at room temperature. Cells do not need to be washed. Add 100 to 150 μl of binding buffer to each sample and analyze by flow cytometry within an hour. It is important to note that it may be difficult to determine the exact percentage of death (Annexin V positivity) from freshly isolated cells, because dead and dying cells are quickly phagocytosed *in vivo*.

2.2.3. Cell viability stains, membrane integrity

7AAD (7-amino-actinomycin D, Molecular Probes, Invitrogen, Eugene, OR, cat. no. A1310) is a membrane-excluded dye that becomes highly fluorescent upon DNA intercalation and thus stains membrane-compromised cells. It can be excited with an argon laser (488 nm), although the

maximum excitation wavelength is 546 nm. It fluoresces red (647 nm) showing minimum overlap with fluorescein or phycoerythrin. It is excluded by viable cells but can penetrate the cell membrane of those that are dead or dying. Wash 0.5 to 1 $\times$ 10^6 cells in FACS buffer (1% FCS, 1 $\times$ PBS, 0.1% NaN_3), centrifuge at 1400 rpm for 5 min, and stain with 7AAD at 10 μg/ml for 15 min at room temperature. Wash and resuspend in FACS buffer before collection on the flow cytometer.

Another compound that measures cell viability is propidium iodide (PI, Sigma, St. Louis, MO, cat. no. P4170). Similar to 7AAD, its fluorescence is substantially enhanced by DNA intercalation, and it is also excluded from viable cells. One significant drawback is that it fluoresces between channels (517 nm), creating background that is difficult to compensate. However, if one chooses to use PI to identify dead cells, it should be added to cells in FACS buffer at a final concentration of 50 μg/ml just before collection by flow cytometry.

Membrane integrity can also be determined by staining cells with trypan blue. Viable cells do not uptake trypan blue; however, the dye easily passes through the membrane of a dead or dying cell, resulting in a blue color. Determining the percentage of live cells excluding trypan blue is called the dye exclusion method. Make a 1:1 mixture of cells and 0.1% trypan blue. Place 10 μl of stained cells on a hemocytometer and allow cells time to settle. By use of a microscope, count the number of unstained live cells and the number of total cells (unstained and stained). Calculate the percentage of viable cells by dividing the number of unstained cells by the total number of cells and multiplying by 100.

3. DNA

3.1. Cell cycle (sub2n)

Another method of determining apoptotic cell death is by quantifying subdiploid levels of DNA, usually visualized by dye intercalation into double-stranded DNA. If cells are permeabilized, 7AAD and PI cause cells to fluoresce in proportion to the amount of DNA per cell (Fig. 10.2). G0/G1 cells have 2n DNA, S/G2 cells have >2n DNA, and apoptotic cells undergoing DNA fragmentation have subdiploid amounts of DNA. These dyes can thus be used to determine the proportion of cells in a population that are in each phase of the cell cycle.

Before staining with 7AAD, 0.5 to 1 $\times$ 10^6 cells need to be suspended in 0.5 ml 150 m*M* NaCl and kept on ice. Add 1.2 ml cold 100% ethanol dropwise while vortexing the cells. Incubate on ice for 30 min before washing cells with PBS. Follow by resuspending in 1% paraformaldehyde (PFA) and 0.01% Tween-20 in PBS. Incubate at room temperature for 30 min then

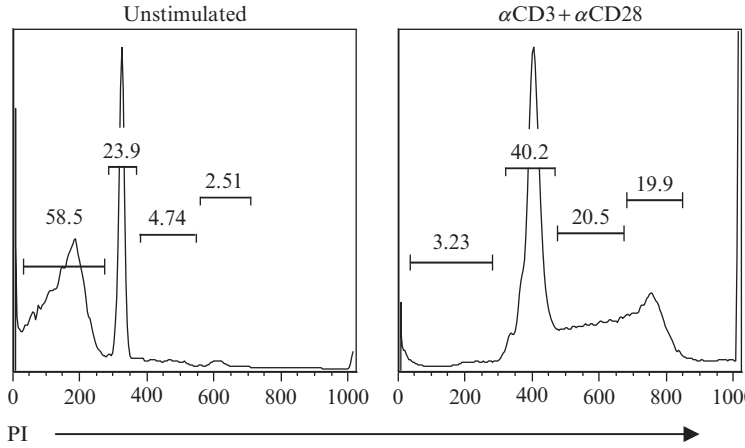

Figure 10.2 **Detection of apoptotic death through measurement of subdiploid DNA. Purified wild-type T cells were cultured in media alone or with anti-CD3 and anti-CD28 for 72 h. Cells were fixed, permeabilized, and stained with propidium iodide. DNA content was measured by passing the cells through a flow cytometer and using a linear scale. Histograms were made by use of the FlowJo software (Tree Star, Ashland, OR).**

wash cells with PBS. Centrifuge fixed cells at 1800 rpm. Incubate another 30 min at room temperature in 100 μl of 380 μ*M* sodium citrate (diluted in PBS) containing 10 μg/ml 7AAD. Wash with PBS, resuspend in FACS buffer and analyze by flow cytometry by use of a linear scale (protocol designed by DR Beisner). To measure cell cycle by staining with propidium iodide, first fix cells with 0.5% PFA on ice for 20 min. Wash with PBS. Next, permeabilize the cells with 0.2% TritonX-100. Wash and resuspend in PI buffer (0.1% TritonX-100, 1 m*M* Tris pH 8.0, 0.1 m*M* EDTA, 0.1% Na citrate, 50 μg/ml PI) with 50 μg/ml RNase A added just before use. Stain cells on ice in the dark for at least 10 min. Wash and resuspend with FACS buffer. Collect on the flow cytometer by use of a linear scale.

3.2. Tunel

A defining characteristic of apoptosis is the cleavage of DNA by endonucleases, resulting in double- and single-stranded breaks (nicks). Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) is a common method used to detect DNA fragmentation (Fig. 10.3).

The enzyme terminal deoxynucleotidyl transferase (TdT) identifies free 3′-OH ends in DNA and catalyzes the template-independent polymerization of deoxyribonucleotides (dUTP). Modified dUTPs (i.e., fluorescein-dUTP) allow for detection by flow cytometry or fluorescence microscopy (*In situ* cell death detection kit, TMR red, Roche, cat. no. 12156792910; FragEL DNA fragmentation detection kit, Fluorescent TdT enzyme,

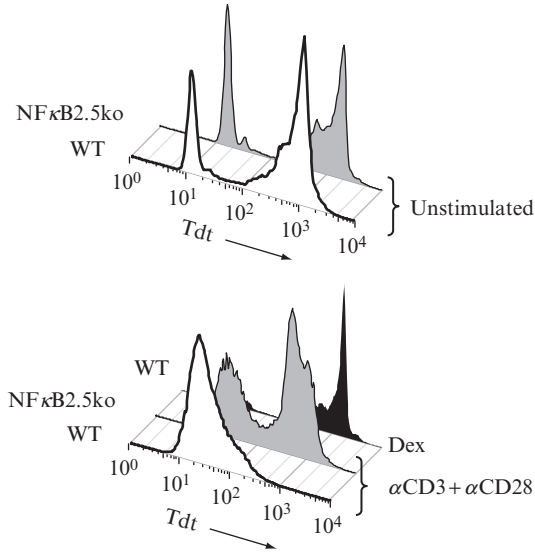

Figure 10.3 Lymphocytes lacking NF-κB die by apoptosis during proliferation. Wild-type (WT) and NFκB2.5ko (*nfkb1*$^{-/-}$*crel*$^{-/-}$*rela*$^{+/-}$) T cells were purified from lymph nodes by negative selection. Cells were cultured in media alone or with anti-CD3 and anti-CD28 for 72 h. Wild-type cells were treated overnight with dexamethasone (Dex) as a positive control for TUNEL staining. Data were collected with a flow cytometer and analysis was done with the Flowjo software.

Calbiochem, cat. no. QIA39.) To allow for the surface staining of cells for flow cytometry, we have derived an alternative fixation and permeabilization protocol when the FragEL DNA fragmentation detection kit is used. Once 0.5 to 1 × 10^6 cells have been washed with FACS buffer, incubate with your desired antibodies conjugated to any fluorophore, except those than fall into the FL1 channel, for 15 min at room temperature. Wash with PBS and fix cells with 1% PFA in PBS for 10 to 20 min at room temperature. Wash and permeabilize with 0.2% Tween-20 in PBS for 10 to 20 min. Wash and resuspend cells in the equilibrium buffer provided in the FragEL DNA kit as described in the kit directions. Follow with TdT labeling for 1 h at 37 °C. The protocol included with the *in situ* cell death kit, TMR red, allows for surface staining before fixation and does not need to be altered. A decent positive control for TUNEL can be obtained by the use of cells cultured with dexamethasone (Sigma, cat. no. D-4902) or the anti-Fas antibody, Jo-2 (BD Pharmingen, cat. no. 554255), overnight.

3.3. Nucleosomal DNA laddering (DNA fragmentation)

The activation of endonucleases preferentially results in the degradation of the internucleosomal linker regions within genomic DNA, producing 180- to 200-base pair DNA fragments (Fig. 10.4). These nucleosomal DNA

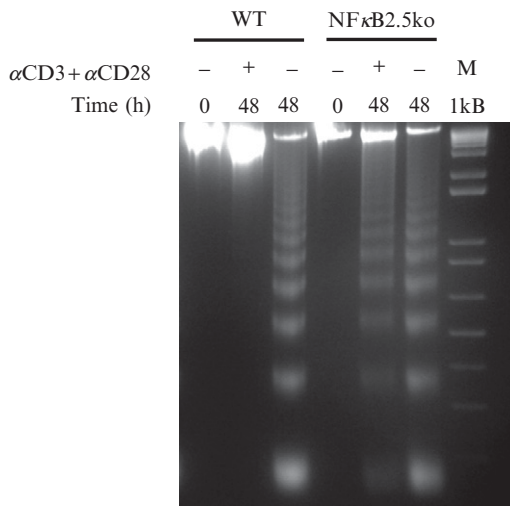

Figure 10.4 Proliferation of NF-κB–deficient T cells results in fragmented DNA. Purified wild-type and NFκB2.5ko (*nfkb1*$^{-/-}$*crel*$^{-/-}$*rela*$^{+/-}$) lymphocytes were stimulated with anti-CD3 and anti-CD28 for 48 h. As a positive control, lymphocytes were also cultured in media alone for 48 h. DNA was prepared as described and resolved on a 2% agarose gel in comparison with a size marker (M), the 1-kb plus ladder.

fragments containing different numbers of nucleosomes appear as a ''ladder'' by gel electrophoresis and are indicative of cells undergoing the late stages of apoptosis (Wyllie *et al.*, 1980).

To access DNA laddering, at least 2 to 5 × 10^6 cells are needed. Harvest, wash and lyse cells with 0.2% TritonX-100 in PBS. Incubate on ice for 10 min. Add RNAseA at 50 μg/ml and incubate for 1 h at 37 °C. Bring cell suspension to a final concentration of 0.5% SDS and 150 μg/ml proteinase K and incubate for 1 h at 50 °C. Add an equal volume of phenol/chloroform, vortex, and centrifuge at 14,000 rpm for 5 min. Recover the aqueous phase and add 0.1 volume of 3 *M* sodium acetate. Use two volumes of ethanol to precipitate the DNA at −20 °C for 1 h. Pellet the DNA by centrifugation, dry, and resuspend in TE buffer before resolving on a 2% agarose gel. Primary cells left in culture with no stimulation for at least 2 days or treated with dexamethasone overnight are sufficient positive laddering controls.

4. Proteins

Many signaling cascades are triggered during the events leading to programmed cell death. For example, the mitochondria could release cytochrome *c*, resulting in the activation of caspase-9 and the caspase-signaling

cascade, ending in the activation of executioner caspases, such as caspases-3 and -7. Likewise, the extrinsic death pathway involving caspase-8 also leads to the activation of executioner caspases. Changes in protein levels of these key players can be measured by immunoblotting.

Prepare cytoplasmic extracts with lysis buffer containing protease and phosphatase inhibitors to prevent protein degradation. Separate 10 to 20 μg of protein by electrophoresis on a SDS-polyacrylamide gel (SDS-PAGE). Transfer to a PVDF membrane and block with 5% milk dissolved in TBST (10 m*M* Tris-Cl pH7.5, 150 m*M* NaCl, 0.25% Tween-20). With gentle agitation, incubate overnight at 4 °C or 2 h at room temperature with primary antibody, diluted 1:100 in 5% milk and 0.02% NaN_3. Caspase antibodies worth mentioning within the intrinsic and extrinsic death pathways are as follows: anti-Caspase-8 clone 1G12, 3B10 (Alexis Biochemicals, cat. no. 804-447-C100, 804-448-C100), cleaved anti-Caspase-3 (BD Pharmingen, cat. no. 559565), pro- and cleaved- anti-Caspase-3 (Cell Signaling Technology, Danvers, MA, cat. no. 9665), and anti-Caspase-9 (Cell Signaling Technology, cat. no. 9504). A few antibodies that detect components in the death receptor (noncanonical) and mitochondrial (canonical) death pathways that lead to apoptosis are as follows: anti-Bim (Sigma, cat. no. B7929), anti-Bax (Cell Signaling, cat. no. 2772), anti-Bcl-xL (Cell Signaling, cat. no. 2762), anti-Bcl-2 (BD Pharmingen, cat. no. 554218), and anti-cytochrome *c* (Cell Signaling Technology, cat. no. 4272). Wash membrane three times with TBST to remove unbound primary antibody, followed by a 1 h incubation with a horseradish peroxidase (HRP)–conjugated secondary antibody to the appropriate species in 5% milk (no NaN_3). Wash membrane and visualize on autoradiography film by use of the ECL system.

5. Metabolic Health

5.1. Mitochondrial membrane potential

Change in the mitochondrial transmembrane potential is one major cause of programmed cell death (Green and Kroemer, 1998). Measuring such a transformation in the cell is one method used to determine the mechanism involved in apoptotic and necrotic death. An increase in membrane permeability results from the mitochondrial permeability transition (MPT) that alters the electrochemical gradient across the membrane, opening the mitochondrial transition pores.

One very useful tool is the JC-1 Mitochondrial Membrane Potential Detection Kit from Molecular Probes (Invitrogen, cat. no. M34152). Similar to TMRE, $DiOC_6$, and rhodamine 123, JC-1 is a fluorescent cationic dye. However, rather than measuring a loss of fluorescence, shifts in mitochondrial polarization can be specifically detected by the color change of JC-1. In viable

cells, the JC-1 dye accumulates in the mitochondria as an aggregate (red). When the mitochondrial potential of the cell collapses, the dye remains in the cytoplasm as a monomer (green). Analysis can be done by flow cytometry or fluorescence microscopy; however, titrations should be done to ensure the collection of accurate data with the least amount of background staining.

5.2. Reactive oxygen species

Under normal physiologic conditions, reactive oxygen species (ROS) are by-products generated in the mitochondria that are quickly removed by antioxidant enzymes. However, when disrupted, such as in instances of environmental stress, the accumulation of ROS can mediate programmed cell death in many cell types (Fiers *et al.*, 1999). Intracellular ROS can be identified by the oxidation of a reduced, cell permeable probe that is naturally nonfluorescent but becomes fluorescent when its acetate groups are cleaved by intracellular esterases.

The ROS detection agent we currently use is the carboxy derivative of fluorescein, carboxy-H_2DCFDA (5-(and-6)-carboxy-2′,7′- dichlorohydrofluorescein diacetate, Molecular Probes, cat. no. C-400); 0.5 to 1 × 10^6 cells are incubated for 30 min at 37 °C in FACS buffer with 5 μM carboxy-H_2DCFDA. Surface marker staining in other fluorophores can be done simultaneously. Samples are washed and centrifuged at 1500 rpm for 5 min. The presence of ROS activity is then measured by flow cytometry. As a positive control, cells can be treated with 100 μM H_2O_2 for 20 h, although it is best to do a titration as each cell type can differ. A few known inhibitors of ROS activity are the reducing agent *N*-acetylcysteine (NAC) and the ROS scavenger butylated hydroxyanisole (BHA).

6. Senescence Detection

Cellular senescence involves an irreversible entering of the G0 phase of the cell cycle concomitant with morphologic changes of the cell. Senescent cells are flat and often damaged when physically manipulated (e.g., trypsinization). Cellular senescence can be brought about by mitochondrial or genotoxic stresses or chronically elevated NF-κB activity, but it is also part of the normal proliferation limit of primary fibroblasts obtained from mouse embryos or human sources. Whereas mouse fibroblasts' proliferative capacity is limited by the elevated oxygen levels of cell culture, human fibroblasts are driven into senescence by shortening telomeres.

One hallmark of senescent adherent cells (such as fibroblasts) is that they are flat. In simple bright-field microscopy, senescent fibroblasts are several times bigger than proliferating fibroblasts and show less birefringence and

contrast. In addition, an endogenous ß-galactosidase with an acidic activity preference is expressed at elevated levels in senescent cells. This senescence-associated ß-galactosidase, or SA-ß-gal, can be revealed by use of X-gal in acidic buffer conditions.

Starting with adherent cells attached to a tissue culture plate, wash cells with PBS at least twice, then fix with 2% (v/v) formaldehyde and 0.2% (w/v) glutaraldehyde in PBS. Wash again with PBS and add 1 ml per 30-mm plate of 5 m*M* potassium ferrocyanide ($K_3Fe(CN)_6$), 2 m*M* $MgCl_2$, 20 m*M* citric acid, 40 m*M* sodium phosphate dibasic (Na_2HPO_4) at pH 6.0 and 1 mg/ml X-gal. Incubate for 2 to 24 h. Blue staining indicates SA-ß-gal expression and is used as a marker for senescence.

Other hallmarks of senescent cells are elevated levels of the cell cycle inhibitor p16. Within a population, p16 levels can be assayed by immunoblot, but standard immunohistochemistry can also be used to reveal p16 levels in individual cells.

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