Aging Cell (2005) **4**, pp217–222 Doi: 10.1111/j.1474-9726.2005.00163.x

Caspase-independent cytochrome c release is a sensitive measure of low-level apoptosis in cell culture models

Joshua C. Goldstein,[1] Francis Rodier,[1] James C. Garbe,[1] Martha R. Stampfer[1] and Judith Campisi[1,2]

[1]Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[2]Buck Institute for Age Research, Novato, CA 94945, USA

Summary

Age-associated loss of tissue function and several chronic diseases may derive in part from the cumulative effects of subtle changes in the level of apoptotic cell death. Because apoptosis is rapid and undetectable once complete, small changes in its incidence are difficult to detect, even in well-controlled cell cultures. We describe a new apoptosis assay that provides greater sensitivity than conventional assays because it measures the accumulation of apoptotic cells. Human and mouse fibroblasts and human mammary epithelial cells that initiated apoptosis were preserved for 3 days by inhibiting caspase activity using the chemical inhibitor Q-VD-OPH (QVD). Cells suspended in the process of apoptosis were scored by immunostaining for cytochrome c, which redistributed from mitochondria in healthy cells to the cytoplasm in dying cells. This caspase-independent cytochrome c release (CICR) assay was more sensitive than several conventional assays when apoptosis was induced by actinomycin D, and detected cumulative background levels of apoptosis over a 3-day interval. Using this assay, we show that normal fibroblasts undergo very little apoptosis upon X-irradiation, indicating dominance of the senescence response in this cell type. Further, apoptosis increased subtly but measurably when human mammary epithelial and skin fibroblast cells entered crisis, indicating that cell death during crisis is largely non-apoptotic.

Key words: aging; cell death; crisis; DNA damage; fibroblasts; mammary epithelial cells.

Introduction

Apoptosis, or programmed cell death, actively eliminates damaged, dysfunctional, virally infected or potentially neoplastic cells from multicellular organisms. Apoptosis is essential for normal metazoan embryogenesis, during which it eliminates excess cells or cells that fail to make proper functional connections. In complex organisms such as mammals, apoptosis is also vital for the maturation of certain tissues, particularly the immune system, and the homeostatic maintenance of adult tissues. Both deficits and surfeits in apoptosis can cause or contribute to pathology, particularly the hyperproliferative and degenerative pathologies associated with aging. In all cases, apoptotic signals engage an intrinsic program of biochemical events that culminates in the degradation of key cellular constituents, including regulatory and structural proteins and genomic DNA. The remaining cell components are sequestered into membrane-bound bodies and engulfed by neighboring cells or macrophages. Thus, apoptosis provides a means to purge tissues of unwanted or defective cells without autolysis and subsequent inflammation (Walker *et al*., 1988).

If deficits or surfeits in apoptosis contribute to mammalian aging, changes in the rate of apoptosis most likely take place slowly and cumulatively over the lifespan (Hasty *et al*., 2003). However, apoptosis is rapid and essentially undetectable once complete. Thus, under most circumstances, the number of cells undergoing apoptosis in a given tissue at a given time is likely small, rendering subtle changes in the incidence or rate of apoptosis difficult to measure. These considerations raise the possibility that the role of apoptosis in aging is underappreciated, both *in vivo* and in cell culture models, due to the technical challenge of detection. Here, we describe a new apoptosis assay that is more sensitive than conventional assays and may help elucidate the role of apoptosis, at least in cell culture models.

Apoptosis is identified by morphological and biochemical changes, including loss of mitochondrial membrane potential ($\Delta\Psi$m), externalization of the plasma membrane lipid phosphatidlyserine, DNA fragmentation and nuclear condensation, and, for adherent cells, detachment from the substratum. These changes result from the activation of caspases, a family of cysteine proteinases (Wolf & Green, 1999). Caspases cleave a number of substrates that initiate or effect many of the changes that identify apoptosis. Conventional apoptosis assays typically measure caspase-dependent events. Thus, caspase-dependent DNA fragmentation can be detected by fluorescence flow cytometry (sub-G1 DNA content assay) (Ormerod *et al*., 1992). Likewise, cells with activated caspases become permeable to the DNA binding dye propidium iodide (PI) (Cotter & Martin, 1996), display phosphatidylserine on the outer plasma membrane leaflet (Koopman *et al*., 1994; Martin *et al*., 1995), and lose $\Delta\Psi$m (Ricci *et al*., 2004), which can be detected by flow cytometery or microscopy.

Two major pathways, termed endogenous and exogenous, activate caspases. The endogenous pathway is generally engaged by cell damage or stress, and results in the translocation of

Correspondence
Judith Campisi, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, 84–171, Berkeley, CA 94720, USA. Tel.: +1 510 486 4416; fax: +1 510 486 4545; e-mail: jcampisi@lbl.gov

Accepted for publication *23 May 2005*

cytochrome c and other proteins from the mitochondrial intermembrane space to the cytosol, with a subsequent drop in $\Delta\Psi$m. Once in the cytoplasm, cytochrome c interacts with the adaptor protein Apaf-1, which recruits and activates caspases in a complex termed the apoptosome (Green & Evan, 2002). Thus, signaling events that trigger the endogenous pathway occur prior to caspase activation and are caspase-independent. The exogenous pathway, by contrast, activates caspases through the binding of physiological ligands to cell-surface death receptors. Ligated death receptors activate upstream caspases, which in turn either activate caspases that effect apoptosis or cleave proteins that initiate the endogenous pathway (Ashkenazi & Dixit, 1998). Thus, cytochrome c release depends on caspase activation by the exogenous pathway, but not the endogenous pathway.

We exploited the caspase-independence of endogenous pathway events to develop a sensitive assay that measures the accumulation of apoptotic cells. The assay uses caspase inhibitors to essentially freeze cells in the apoptotic process after mitochondrial cytochrome c release but before cell destruction. This caspase-independent cytochrome c release (CICR) assay detected small changes in apoptosis incidence in cell cultures, and should facilitate understanding how chronic cellular damage or stress influence aging phenotypes by subtly altering the apoptotic response.

Results

Conventional assays provide only a snapshot of the incidence of apoptosis in cell populations. We reasoned that low levels of apoptosis could be amplified by measuring the accumulation of apoptotic cells. To test this idea, we treated subconfluent cultures of primary mouse embryo fibroblasts (MEFs), normal human embryo fibroblasts (WI-38), and normal human mammary epithelial cells (HMECs) with the caspase inhibitors zVAD or QVD and/or actinomycin D (Act-D; 40 nM). Act-D induces cytochrome c release whether or not caspase inhibitors are present (Goldstein *et al.*, 2000). Initially, we scored for cell detachment, which would hinder cytochrome c immunostaining. As expected, Act-D caused all three cell types to adopt a characteristic apoptotic morphology and detach from the culture dish after 3 days. zVAD and QVD prevented detachment to varying degrees, depending on the cell type. For example, 100 M zVAD failed to prevent detachment of MEFs, but 40 M QVD largely prevented detachment under the same conditions (Fig. 1). HMECs behaved similarly to MEFs in that QVD was more effective than zVAD in preventing cell detachment (data not shown). Act-D-treated WI-38 cells, by contrast, remained equally well attached whether the inhibitor was zVAD or QVD (data not shown). In either case, although cells treated with Act-D and caspase inhibitors remained attached for 3 days, they did not proliferate. Failure to proliferate was not due to the caspase inhibitors *per se* because MEFs, HCA-2 or HMEC treated with the inhibitors alone proliferated for 5 days at the same rate as cells treated with vehicle (DMSO) or unadulterated growth medium (supplementary Fig. S1). These findings suggested that QVD stalled, at least temporarily, Act-D-induced detachment of MEFs, human fibroblasts and HMECs.

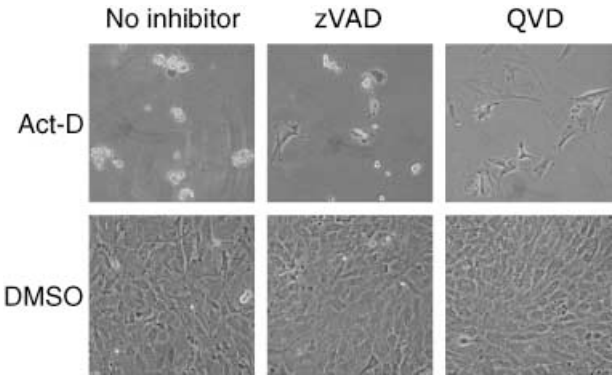

Fig. 1 QVD preserves adhesion of Act-D-treated cells. MEFs were given Act-D (40 nM in DMSO) or DMSO and either no inhibitor, zVAD (100 M) or QVD (40 M), as indicated. After 3 days, cells were photographed at 10× magnification.

To test this idea more directly, we treated MEFs with Act-D in the absence or presence of QVD and measured apoptosis by standard assays. Cells varied in their sensitivity to protection by QVD. For example, QVD protected some cell types, such as HeLa, from Act-D-induced detachment for as long as 6 days after cytochrome c release (supplementary Fig. S2A,B). We chose a 3-day interval because QVD protected > 90% of Act-D-treated MEFs from detachment for 3 days, after which cell viability declined steadily (supplementary Fig. S2A). We used three standard apoptosis assays, all of which employ flow cytometry for detection (Fig. 2). These were the appearance of a sub-G1 DNA peak (Fig. 2A), reduction in $\Delta\Psi$m (Fig. 2B), and ability to exclude PI and its subsequent binding to nuclear DNA (Fig. 2C). In each case, manifestations of apoptosis were apparent 1–2 days after addition of Act-D, and were substantially reduced by 40 M QVD.

We next examined Act-D-treated MEFs for diffuse cytochrome c immunostaining, indicative of release from mitochondria (Fig. 3A). In contrast to the other indicators of apoptosis, which declined in the presence of QVD, QVD increased the fraction of Act-D-treated cells with diffuse cytochrome c staining. After 3 days, 96% of cells treated with Act-D and QVD showed cytoplasmically distributed cytochrome c. In the absence of QVD, very few cells remained attached. Of those, 44% showed diffuse cytochrome c immunostaining (Fig. 3A, supplementary Table S1). Thus, in the absence of QVD, apoptosis proceeded until eventual cell detachment and/or disintegration. QVD temporarily blocked apoptosis, allowing cells to remain attached until other forms of cell death occur. These findings suggested that the combined use of QVD and cytochrome c immunostaining might permit the accumulation and detection of cells in the act of apoptosis over an extended interval (at least 3 days). We termed this assay the caspase-independent cytochrome c release (CICR) assay.

Comparisons between CICR and standard apoptosis assays showed the CICR assay is more sensitive. The sub-G1 peak

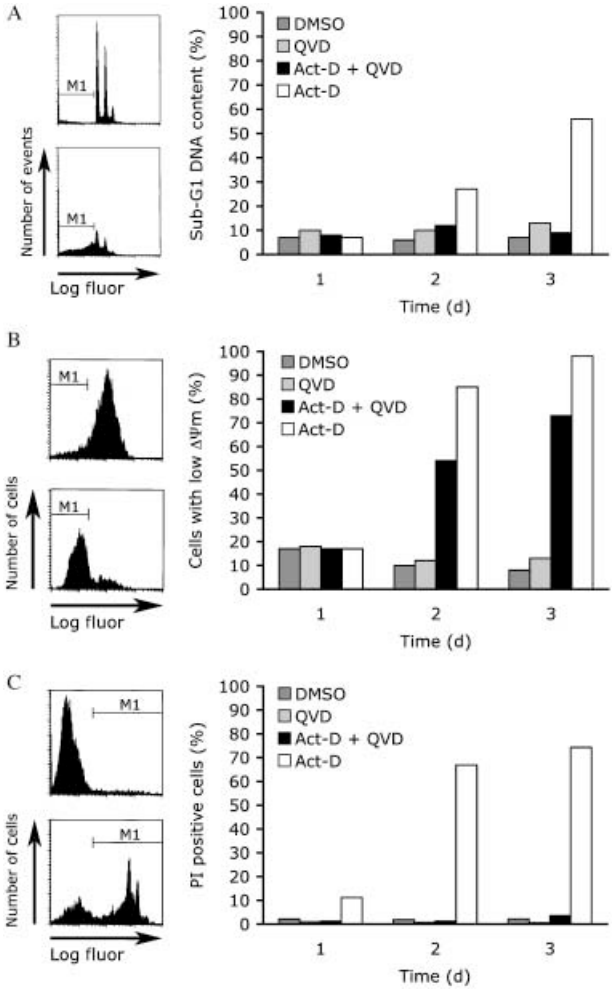

Fig. 2 Comparison of apoptosis assays. Sub-G1 peak (A), mitochondrial membrane potential (B) and propidium iodide exclusion (C) assays were performed using MEFs treated with Act-D or DMSO in the presence or absence of QVD. The cells were assayed every 24 h for three consecutive days. Shown is one of two experiments that gave similar results. (A) Sub-G1 peak FACS measurements. M1 (left part of the histograms) shows the gate used to detect apoptotic cells in cultures treated with DMSO (top) or Act-D (bottom) after 3 days. A time course of the fraction of cells in the M1 compartment is shown on the right. (B) FACS analysis of mitochondrial membrane potential in living cells. M1 (left part of the histograms) shows the gate used to score cells with low membrane potential in DMSO-(top) or Act-D-treated (bottom) cultures after 2 days. The time course of the fraction of cells in the M1 compartment is on the right. (C) FACs analysis of propidium iodide exclusion. M1 (right part of the histograms) shows the gate used to detect cells that fail to exclude PI in DMSO-(top) and Act-D-treated (bottom) cultures. The time course is shown on the right.

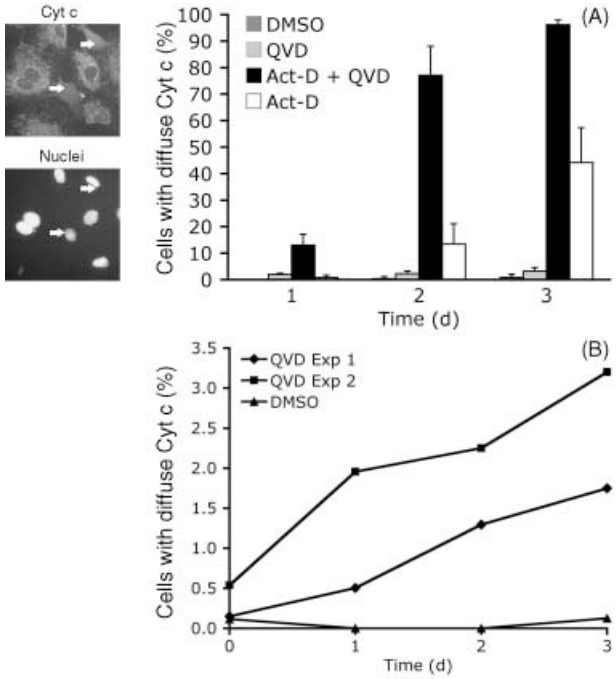

Fig. 3 CICR assay. (A) Cumulative apoptosis induced by Act-D or normal culture conditions (basal apoptosis). MEFs were scored for diffuse cytochrome c after treatment with DMSO or Act-D for 1–3 days in the presence or absence of QVD. Images (left, 20×) show representative cells after 1 day. The arrows show diffuse cytochrome c staining (left, top) and nuclear staining (left, bottom). A time course of cells with diffuse cytochrome c staining is shown on the right. Approximately 20–750 cells were counted per condition (Table S1). Error bars are SD, $n = 4$ fields. (B) CICR measures basal apoptosis over a 3-day interval. MEFs were cultured in the presence of QVD or DMSO for 3 days and assayed daily for apoptotic cells using the CICR assay. Between 600 and 800 cells were scored for each data point of the two independent QVD experiments and DMSO control.

determination and loss of $\Delta\Psi$m assays detected little or no apoptosis in MEFs treated with Act-D for 1 day (Fig. 2A,B). However, the CICR assay showed 13% of cells were apoptotic at that time (Fig. 3A). Data from the standard assays could be interpreted to mean that Act-D starts killing MEFs 24–48 h after exposure. However, the CICR assay showed that Act-D begins killing cells within the first 24 h. Further, the standard PI exclusion assay indicated only 74% of Act-D-treated cells died after 3 days, but the CICR assay indicated 96% of the cells were dead or dying after 3 days (Figs 2C and 3A). We conclude that the CICR assay scores a higher incidence of apoptosis because cytochrome c release precedes nuclear fragmentation and PI positivity, and conventional assays cannot measure cells that complete the apoptotic program and disintegrate.

To explore the sensitivity of the CICR assay, we measured background levels of apoptosis in untreated MEFs. We reasoned that, if the assay detects cumulative apoptotic events, it should show a progressive increase in positive cells over time. Indeed, the assay detected low-level background apoptosis cumulatively and progressively in two different MEF cultures over 3 days, at which time 2–3% of the cells were dead or dying (Fig. 3B). None of the conventional assays we used could reliably measure these low levels of apoptosis (Fig. 2A–C). Moreover, the conventional assays could not measure accumulated apoptotic cells (Fig. 2A–C).

X-irradiation induces a senescent growth arrest in cultured human fibroblasts (DiLeonardo *et al.*, 1994). We used the CICR assay to determine whether X-irradiation also induced apoptosis, albeit in only a few cells. We irradiated (10 Gy) human fibroblasts, immediately added QVD, and then used the CICR assay to detect apoptosis 3 days later. Apoptotic cells remained rare in the irradiated population, reaching only about ~1% over the 3 days following irradiation (Fig. 4).

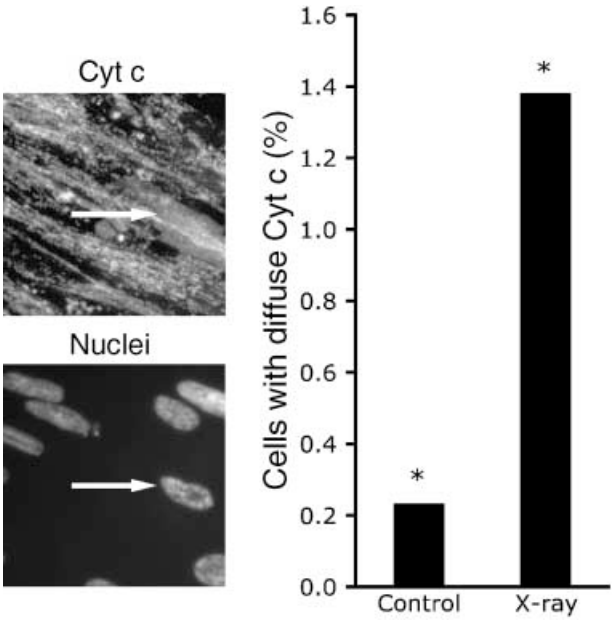

Fig. 4 Measurement of apoptosis by CICR in X-irradiated human fibroblasts. Proliferating WI-38 fibroblasts were untreated (Control) or X-irradiated with 10 Gy (X-ray) and assayed for 3 days by CICR. Arrow shows an apoptotic cell identified by diffuse cytochrome c staining (left, top) and the corresponding nucleus (left, bottom). The numbers of cells scored were 362 (Control) and 428 (X-ray). **P* = 0.049 in an unpaired single tailed Student's *t*-test, *n* = 8 fields.

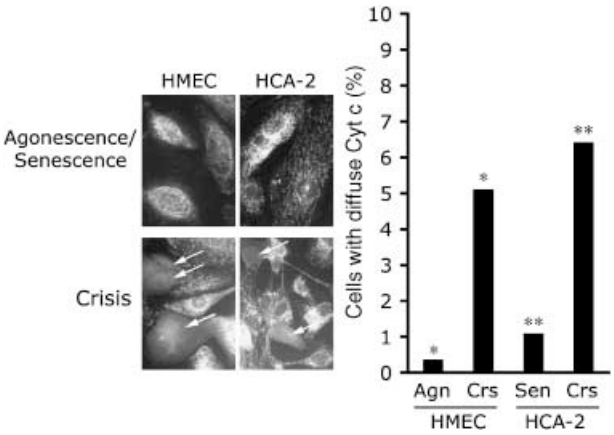

Fig. 5 CICR assay detects increased apoptosis in cells in crisis. HMECs and HCA-2 fibroblasts were cultured to the end of their replicative lifespan, agonescence/senescence, and induced into crisis by p53 inactivation (HMECs) or p53 inactivation and E7 expression (HCA-2). The cells were assessed for apoptosis by CICR for a 3-day period. Arrows identify apoptotic cells (diffuse cytochrome c staining, bottom). The numbers of cells scored were 247 (agonescence, Agn), 465 (HMEC crisis, Crs), 343 (replicative senescence, Sen) and 484 (HCA-2 crisis, Crs). **P* = 0.01 and ***P* = 0.02 in an unpaired single tailed Student's *t*-tests, *n* = 5 fields.

Finally, we used the CICR assay to determine apoptosis levels in HMECs and HCA-2 fibroblasts in crisis. HMECs frequently silence the pRB activator p16; such cells undergo a telomere length-dependent replication block known as agonescence (Romanov *et al*., 2001). Agonescence can be temporarily overcome by loss of p53 function (Stampfer *et al*., 2003). Likewise, human fibroblasts with defective p53 and pRB function temporarily bypass the telomere length-dependent replication block known as senescence (Shay *et al*., 1991). In both cases, telomeres eventually become very short and hence unstable, whereupon cells enter a state known as crisis. Cells die gradually but almost inevitably during crisis, but it is not known whether they die by apoptosis.

We used the CICR assay to measure apoptosis in HMECs that either arrested proliferation at agonescence or entered crisis owing to expression of the genetic suppressor element GSE22, which inactivates p53 (Ossovskaya *et al*., 1996). We also measured apoptosis in human fibroblasts (HCA-2) that were either senescent or driven to crisis by expression of GSE22 and E7, which inactivates pRB (Shay *et al*., 1991). Over 3 days, apoptosis occurred in < 1% of agonescent HMECs and ~1% of senescent fibroblasts (Fig. 5) These findings are consistent with reports that apoptosis, as measured by Annexin V binding, is rare in agonescent cultures (Romanov *et al*., 2001); they also indicate the stability of senescent cultures. As expected, these cultures had very few proliferating cells, as determined by BrdU incorporation in agonescent (5.6%, 1 day incubation) or sennescent (5%, 3-day incubation) cultures. By contrast, ~5% of HMECs and HCA-2 cells in crisis underwent apoptosis over 3 days (Fig. 5). Consistent with these cultures being in crisis (Wei & Sedivy, 1999), 25% of HMECs and 23% of HCA-2 incorporated BrdU during 1 or 3-day intervals respectively. Importantly, cell number did not increase. Given this high labeling index, the relatively low rate of apoptosis cannot alone explain the gradual loss of cells from cultures in crisis, suggesting other modes of cell death (e.g. necrosis, mitotic catastrophe) occur during crisis.

Discussion

Background or basal rates of apoptosis likely change during mammalian aging (Suh *et al*., 2002). Such changes may contribute to the loss of tissue structure and function, as well as hyperplastic and degenerative diseases, which are hallmarks of aging (Joaquin & Gollapudi, 2001; Zhang & Herman, 2002; Campisi, 2003). The number of cells undergoing apoptosis at any time is likely to be small, and rare apoptotic cells are difficult to quantify. We describe here a method to measure apoptosis cumulatively over 3 days. Although we did not test this assay in animals, we show it is sensitive and useful for measuring rare apoptotic events in cell cultures.

The CICR assay is based on quantifying cells that release cytochrome c from mitochondria to the cytosol. A major strength of this assay is its ability to integrate apoptotic events over a 3-day period. In addition, the CICR assay is more sensitive than several conventional apoptosis assays. However, the CICR assay cannot detect cells induced to die by signals that do not engage mitochondria or do so via caspase-dependent pathways. Nonetheless, the CICR assay successfully measured low levels of apoptosis in untreated MEF cultures, X-irradiated human fibroblast cultures, and HMEC and fibroblast cultures at agonescence, senescence and crisis.

Fibroblasts respond to X-irradiation by a permanent growth arrest known as senescence (DiLeonardo *et al*., 1994), although other cell types, such as lymphocytes, respond by apoptosis (Boreham *et al*., 1996). The CICR assay showed that 1% of X-irradiated fibroblasts undergo apoptosis, raising the possibility that a minority of isogenic fibroblasts activate the apoptotic response, perhaps by modifying the activity or balance of Bcl-2 family members (Oltvai *et al*., 1993; Deverman *et al*., 2002).

Crisis is a state of genomic instability that occurs in culture and *in vivo* (Artandi & DePinho, 2000). Cell populations in crisis show the simultaneous occurrence of cell proliferation, death and cell cycle arrest (senescence), with cell number gradually declining over time (Hara *et al*., 1991; Shay *et al*., 1991; Wei & Sedivy, 1999). Crisis poses a barrier to the development of cancer (Artandi & DePinho, 2000), as does senescence (Campisi, 2001). Thus, it is not surprising that populations in crisis contain senescent cells (Wei & Sedivy, 1999; Shay & Wright, 2004). We used the CICR assay to show that apoptosis, which is also a potent tumor-suppressive mechanism (Green & Evan, 2002), is similarly up-regulated during crisis.

We expect the CICR apoptosis assay will aid in explaining a number of biological phenomena that can be modeled in culture and exhibit low but potentially important levels of apoptosis.

Experimental procedures

CICR assay

We plated cells in four-well chamber slides (Nalge Nunc International, Naperville, IL, USA), generally 3×10^4 per well, and added Act-D (40 nM), DMSO (0.2%), QVD (40 M) or QVD plus Act-D for 1, 2 or 3 days. QVD (and Act-D + QVD) was replaced each day. At the end of the experiment, cells were washed twice with PBS and fixed with 4% formaldahyde in PBS for 5 min at room temperature. Fixed cells were washed twice with PBS and incubated in blocking buffer (3% BSA and 0.1% Triton-X in PBS) for 30 min at room temperature. Cells were incubated for 1 h at room temperature with cytochrome c antibody (Pharmingen, catalog # 556432) diluted 1 : 800 in blocking buffer containing 15 g mL^{-1} Hoechst 33342, washed five times with PBS, and incubated with anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA; catalog # A21121) diluted 1 : 800 for 1 h at room temperature. After washing five times with PBS, cells were mounted in Prolong (Molecular Probes).

Cells were scored as apoptotic if they met the following criteria: (1) presence of diffuse cytochrome c staining in both the cytoplasm and the nucleus; (2) lack of reticulated or punctate staining in the cytoplasm. Typically, images were acquired using a 20× objective, and 500–800 cells were scored. For the exact number of cells counted in Fig. 3(A), see Table S1. Nuclei were counted using images of uniformly illuminated Hoechst staining imported into Image J (http://rsb.info.nih.gov/ij/).

Significance was determined using unpaired single tailed Student's *t*-tests and error bars designate standard deviations.

Cell culture and reagents

Mouse embryo fibroblasts (MEFs) were derived and cultured in 3% oxygen as described (Parrinello *et al*., 2003). WI-38 and HCA-2 fibroblasts were obtained and cultured as described (Itahana *et al*., 2002; Itahana *et al*., 2003). Post-selection human mammary epithelial cells (HMECs) were derived and cultured as described (Hammond *et al*., 1984). As described, cells were infected with retroviruses expressing either GSE-22 (Itahana *et al*., 2002) or E7 (Itahana *et al*., 2003). Act-D and propidium iodide (PI) were from Sigma-Aldrich, zVAD-FMK and QV-D-OPH (QVD) were from MP-Biomedicals (Aurora, OH, USA), and tetramethylrhodamine ethyl ester (TMRE) and Hoechst 33342 were from Molecular Probes.

Cell death assays

Nuclear fragments containing a sub-G1 DNA content, and cells capable of excluding PI, were quantified by flow cytometery, essentially as described (Cotter & Martin, 1996). Relative mitochondrial membrane potentials were measured by staining cells with TMRE (50 nM) and measuring fluorescence by flow cytometry, as described (Waterhouse *et al*., 2001).

Supplementary material

The following supplementary material is available for this article online on the Blackwell Synergy website:

Fig. S1 QVD does not inhibit growth. HCA-2 fibroblasts and HMEC were grown in normal growth media (DMEM, MEGM) or normal growth media supplemented with 0.1% DMSO (DMSO) or 0.1% DMSO + 40 M QVD (QVD). Growth curves were averaged triplicates (HCA-2) or duplicates (HMEC) and error bars (HCA-2) are SD.

Fig. S2 Attachment of cells with diffuse cytochrome c in the presence of QVD. (A) MEFs and HeLa cells were grown in media containing Act-D (MEFs 40 nM, HeLa 200 nM) and QVD for 8 days. Media and drugs were replaced daily. Each data point is the average of four cells counts and error bars are SD. (B) A CICR analysis of HeLa cells treated with Act-D. HeLa were scored for diffuse cytochrome c after treatment with Act-D (200 nM) for 0–3 days in the presence QVD. Images (left, 20×) show cytochrome c (top) and nuclei (bottom) after 1 day. Arrows mark cells that have not released cytochrome c. Quantification is on the right. n = 780 (Day 0), 790 (Day 1), 410 (Day 2) and 251 (Day 3) cells.

Table S1 Raw data from Fig. 3(A).

Acknowledgments

We thank Campisi laboratory members for helpful advice and thought-provoking discussions. This work was supported by a National Institutes of Health National Research Service Fellowship to J.C.G. (AG24015), National Institute on Aging (AG17242)

and Department of Defense (DAMD17-03-1-0546), grants to JC, and Department of Defense grant (DAMD17-02-1-0443), all with support, and under contract AC03-76SF00098 to the University of California from the US Department of Energy.

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