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Mitochondrial DNA damage induces apoptosis in senescent cells

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Senescence is a cellular response to damage and stress. The senescence response prevents cancer by suppressing the proliferation of cells with a compromised genome and contributes to optimal wound healing in normal tissues. Persistent senescent cells are also thought to drive aging and age-associated pathologies through their secretion of inflammatory factors that modify the tissue microenvironment and alter the function of nearby normal or transformed cells. Understanding how senescent cells alter the microenvironment would be aided by the ability to induce or eliminate senescent cells at will in vivo. Here, we combine the use of the synthetic nucleoside analog ganciclovir (GCV) with herpes simplex virus thymidine kinase (HSVtk) activity to create or eliminate senescent human cells. We show that low concentrations of GCV induce senescence through the accumulation of nuclear DNA damage while higher concentrations of GCV, similar to those used in vivo, kill non-dividing senescent cells via mitochondrial DNA (mtDNA) damage and caspase-dependent apoptosis. Using this system, we effectively eliminated xenografted normal human senescent fibroblasts or induced senescence in human breast cancer cells in vivo. Thus, cellular senescence and mtDNA damage are outcomes of synthetic nucleoside analog treatment, indicating that the GCV–HSVtk combination can be used effectively to promote the targeted formation or eradication of senescent cells.

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Cellular senescence suppresses cancer by halting the proliferation of damaged cells that are at risk for malignant progression. ¹–⁴ First formally described in the 1960s, ⁵ cellular senescence has been linked to two important phenomena in complex organisms: tumor suppression and aging. It is now clear that cellular senescence is an essential tumor-suppressive mechanism, rivaling apoptosis in importance. ¹, ² More recently, the senescence response was shown to participate in tissue repair and occurs after DNA-damaging cancer therapies, potentially contributing to the success and side effects of these treatments. ⁴, ⁶–⁸

The senescence growth arrest is tumor suppressive, because it prevents the expansion of cells that are potentially oncogenic. How cellular senescence contributes to aging or tissue repair remains somewhat unclear. The senescence response might drive aging by depleting progenitor cell pools. Additionally, the ability of senescent cells to secrete inflammatory molecules might contribute to tissue repair, but persistent senescent cells might drive chronic inflammation, a major risk factor for age-related pathologies. ⁵ Most senescent cells express the p16⁰INK₄a tumor-suppressor protein, which also increases with age in mouse and human tissues. ⁹–¹¹ p16⁰INK₄a expression correlates well with other senescence markers, such as senescence-associated beta galactosidase (SA-β-gal) activity. ¹² The expression of both biomarkers often coincides in vivo in mice and humans. ⁹, ¹¹ Based on the expression of these two biomarkers, there is substantial evidence that senescent cells accumulate with age in vertebrate tissues; moreover, senescent cells are evident at sites of numerous age-related pathologies, such as atherosclerosis, osteoarthritis and precancerous lesions. ¹³–¹⁸

Recent data show that most senescent cells eventually disappear in mice, with evidence of tissue infiltration by innate immune cells, suggesting that senescent cells are cleared by the immune system. ¹⁹–²¹ Similarly, senescent cells induced during wound repair appear to be cleared by the immune system. ²², ²³ Nonetheless, senescent cells accumulate with age and pathology and are presumed to resist immune clearance by as yet unknown mechanisms. Importantly, in a mouse model of accelerated aging, elimination of senescent cells prevented certain late-life pathologies, lending strong credibility to the hypothesis that senescent cells contribute to aging phenotypes. ²⁴

Understanding the nature and functions of senescent cells during wound repair, cancer therapy and aging requires new tools to modulate and visualize the senescence response in tissues. When targeted to senescent cells, for example, using senescence-associated promoters, these tools could allow the detection of senescent cells in tissues and living mice and/or their specific induction or elimination in a

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Abbreviations: 3MR, trimodal reporter-killer fusion protein; CICR, Caspase-Independent Cytochrome c Release; DNA-SCARS, DNA segments with chromatin alterations reinforcing senescence; GCV, ganciclovir; HSVtk, herpes simplex virus thymidine kinase; mtDNA, mitochondrial DNA; RFP, red fluorescent protein; rLUC, Renilla luciferase; SA- β-gal, senescence-associated beta galactosidase

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regulated fashion. Recently, mouse models were developed in which the p16\textsuperscript{INK4a} promoter drove expression of a transgene that caused apoptosis or luciferase expression in senescent cells in vivo.\textsuperscript{24,25}
was determined as described in Figure 1d after plating for 7 days (media + GCV). Light gray: HCA2-control cells; dark gray: HCA2-3MR cells. Cells were seeded on glass slides and given the indicated concentrations of GCV 48 h
determined in at least 200 cells per GCV concentration. (c) Persistent DNA damage foci were determined in cells treated with GCV. Light gray: HCA2-3MR cells treated with GCV for 9 days; dark gray: HCA2-3MR cells treated similarly and chased for 3 days in GCV-free media. Cells on glass slides were given the indicated concentrations of GCV 48 h after plating for 9 or 12 days (media + or − GCV were replenished every 2 days). The percentage of cells with ≥3 persistent 53BP1 nuclear foci (DNA-SCARS) was determined as described in Figure 1d

Figure 2  GCV induces senescence through DNA damage accumulation. (a) Top panel: HCA2-3MR cells were given the indicated concentrations of GCV 48 h after plating; media + or − GCV were replenished every 2 days. Cells were fixed and SA-β-Gal staining (blue) was performed 10 days later. Lower panel: The fraction of SA-β-Gal-positive cells was quantified and plotted as the percentage of positive cells per GCV concentration. (b) EdU (5-ethyl-2'-deoxyuridine) DNA labeling indexes were determined in cells treated with GCV. Light gray: HCA2-control cells; dark gray: HCA2-3MR cells. Cells were seeded on glass slides and given the indicated concentrations of GCV 48 h after plating for 7 days (media + or − GCV were replenished every 2 days). EdU was added from day 7 to day 8 (24 h pulse) and the percentage of EdU-positive cells was determined in at least 200 cells per GCV concentration. (c) Persistent DNA damage foci were determined in cells treated with GCV. Light gray: HCA2-3MR cells treated with GCV for 9 days; dark gray: HCA2-3MR cells treated similarly and chased for 3 days in GCV-free media. Cells on glass slides were given the indicated concentrations of GCV 48 h after plating for 9 or 12 days (media + or − GCV were replenished every 2 days). The percentage of cells with ≥3 persistent 53BP1 nuclear foci (DNA-SCARS) was determined as described in Figure 1d

As another step toward developing approaches to study/target senescent cells, we show here that ganciclovir (C9H5N13O4 (GCV) and the herpes simplex virus thymidine kinase (HSVtk) can be used to both induce and kill senescent cells in vivo. GCV is a synthetic nucleoside analog widely used to treat herpes viral infections in humans. The viral thymidine kinase binds GCV with high affinity and converts it to a toxic nucleotide after incorporation into the viral and nuclear DNA of infected cells, thereby promoting apoptotic cell death. The usefulness of the GCV–HSVtk combination has a long history in cancer gene therapy and is currently a potential therapeutic tool when incorporated into oncolytic viruses.

Here we show that, in response to low doses of GCV, normal and cancer cells expressing HSVtk undergo senescence due to nuclear DNA damage. In response to higher GCV doses, normal and senescent cells expressing HSVtk undergo apoptosis as a consequence of mitochondrial DNA (mtDNA) damage. Thus, the GCV–HSVtk combination can be used as a multifunctional inducer or killer of senescent cells.

Results

GCV induces nuclear DNA damage and cell death in human fibroblasts. We used a lentiviral vector to constitutively express the trimodal reporter-killer fusion protein (3MR) in primary human foreskin fibroblasts (HCA2). 3MR contains monomeric red fluorescent protein (mRFP), Renilla luciferase (rLUC), and a functional fragment of the HSVtk. Following infection and selection, 3MR expression was confirmed by mRFP fluorescence and rLUC immunofluorescence (Supplementary Figure S1).

With increasing concentrations of GCV, 3MR-expressing cells reduced proliferation or cell number, proportional to the dose of GCV (0–1 μg/ml) (Figure 1a). At low doses (0.001–0.01 μg/ml), GCV retarded or arrested proliferation. At higher doses (≥0.1 μg/ml), GCV caused cell loss (Figure 1a). Cell loss entailed an accumulation of cells with a sub-G1 DNA content (Figure 1b), suggesting apoptotic cell death. We confirmed apoptosis using Annexin V—DAPI live-dead staining, which showed increased numbers of early and late apoptotic cells (Figure 1c). Intermediate GCV doses (0.01–0.1 μg/ml) increased DNA double-strand breaks, as measured by nuclear 53BP1 DNA damage foci, detectable by immunofluorescence (Figure 1d). 3MR-expressing cells but not in control cells in which GCV was non-toxic up to ~10 μg/ml (Figures 1d–f and Supplementary Figure S2).

GCV induces senescence and persistent DNA damage foci. Low concentrations of GCV (0.01 μg/ml) induced cellular senescence in nearly all 3MR-expressing fibroblasts, as determined by an absence of cell loss (Figure 1a) and a dose-dependent increase in the percentage of cells expressing SA-β-gal activity (Figure 2a). At higher concentrations (0.1–1 μg/ml), most cells detached from the culture dish (Figures 1a and b), although the few cells that remained attached were almost all SA-β-gal-positive (Figure 2a). Moreover, 3MR-expressing cells treated with GCV failed to replicate DNA (Figure 2b), as expected of growth-arrested senescent cells. Further, 53BP1 foci induced by sustained...
GCV treatment of 3MR-expressing cells were persistent, a feature of DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) and a hallmark of senescent cells. Thus, removal of GCV for 3 days following an initial 9-day treatment did not reduce the number of 53BP1 foci, supporting the conclusion that GCV can induce senescence in 3MR-expressing cells, with accompanying persistent DNA-SCARS (Figure 2c). Finally, GCV-induced senescence in 3MR-expressing cells entailed increased levels of SA-β-gal, as well as increased levels of the p53-regulated cell-cycle inhibitor p21WAF1 (Supplementary Figures S3A and B).

GCV induces caspase-dependent apoptosis in senescent cells. Upon phosphorylation by HSVtk, GCV is known to kill dividing cells by incorporating into nuclear DNA, causing replication-dependent double strand breaks. However, doses of GCV between 0.1 and 1 μg/ml induced senescence (growth arrest) of surviving 3MR-expressing cells (Figures 1 and 2), these growth-arrested cells continued to die over longer intervals (Figure 1a).

To directly test whether GCV could kill non-dividing senescent cells, we induced senescence in control and 3MR-expressing cells using X-irradiation (10 Gy; Supplementary Figure S3), and measured the number of senescent cells that remained attached to the culture dish following GCV treatment (Figure 3a). Despite their non-dividing status, senescent 3MR-expressing cells showed substantial detachment between 4 and 9 days following GCV treatment. Similarly to proliferating cells, GCV killed these senescent cells by apoptosis, as determined by Annexin V—DAPI live-dead staining (Figures 3b and c).

To confirm apoptotic cell death, we used the sensitive CICR (Caspase-Independent Cytochrome c Release) assay that prevents late-stage caspase-dependent apoptosis progression, thus allowing apoptotic cells to accumulate over several days.
days. Following simultaneous treatment with GCV and a broad caspase inhibitor, accumulated cells attempting to undergo apoptosis were scored by immunofluorescence for diffuse cytoplasmic cytochrome c released from the mitochondria (as opposed to punctate mitochondrial cytochrome c in non-apoptotic cells) (Figure 3d). CICR quantification showed that 10 μg/ml GCV induced apoptosis in ~50% of senescent cells over a 4-day interval following GCV exposure (Figure 3e). Thus, GCV induced caspase-dependent cell death in senescent cells.

GCV incorporates into the mtDNA and causes mtDNA damage. Senescent fibroblasts do not synthesize nuclear DNA but have an increase in mitochondrial mass.\(^\text{38}\) We reasoned that GCV might kill senescent cells by S-phase-independent incorporation into mtDNA, as suggested by anti-cancer strategies using HSVtk.\(^\text{39–41}\) To test this possibility, we incubated senescent and non-senescent cells, either control or 3MR-expressing, with 0.1 μg/ml tritiated GCV for 48 h. Radioactivity was detected in the mtDNA of 3MR-expressing cells, whether dividing (non-senescent) or non-dividing (senescent) (Figure 4a). To determine whether GCV damaged mtDNA in 3MR-expressing cells, we analyzed mtDNA by agarose gel electrophoresis. We detected intact mtDNA (16.5 kb) in control and 3MR-expressing senescent cells. However, only in GCV-treated 3MR-expressing senescent cells, we detected smaller (damaged) molecules that migrated faster (Figure 4b).

GCV eliminates senescent 3MR-expressing cells in vivo. To determine whether the GCV–HSVtk combination could eliminate senescent cells in vivo, we took advantage of the rLUC in 3MR to track the fate of 3MR-expressing cells in mouse xenografts. We induced senescence in 3MR-expressing HCA2 cells by X-irradiation (10 Gy) and, 3 days later, injected 5 × 10⁶ cells into the right flank of nu/nu mice. One day later, we administered PBS (vehicle control) or GCV (25 mg/kg) for 10 days and measured luminescence at regular intervals thereafter (Figure 5a). In the absence of GCV (vehicle alone), luminescence declined between 6 and 10 days, presumably due to clearance by the innate immune system, as reported.\(^\text{22,42}\) GCV, however, eliminated senescent cells significantly faster, with 50% clearance by 6 days and complete clearance by 8 days (Figure 5a).

GCV induces senescence in 3MR-expressing breast cancer cells in vivo. We used a lentivirus to express 3MR in a human breast cancer cell line, MDA-MB231-D3H2LN, which also expresses firefly luciferase. In culture, high-dose GCV (2 and 20 μg/ml) eliminated >95% of cells within 5 days (Figure 5b). GCV was shown to efficiently reduce HSVtk-harboring cancer cells in vivo.\(^\text{43}\) Similarly, GCV treatment at the time of injection of 3MR-expressing MDA-MB231-D3H2LN cells (GCV d0) prevented tumor formation (Figure 5c). Moreover, GCV treatment 10 days after cancer cell injection (GCV d10) slowly shrank established tumors (Figure 5c), suggesting not all cancer cells underwent rapid apoptosis but rather some underwent senescence. Indeed, tumors treated with GCV at d10 and collected 21 days after injection had a significant percentage of senescent cells.

Discussion

Nucleoside analogs such as GCV are used to target cytomegaloviruses and herpes viruses.\(^\text{26}\) Drug specificity is achieved because the viral TK has a much higher affinity for GCV and similar analogs than the cellular TK. TK phosphorylates GCV, and, upon further phosphorylation by cellular enzymes, the drug competes with deoxyguanosine triphosphate for incorporation into nascent DNA chains, leading to DNA synthesis termination. In the presence of HSVtk, GCV not only prevents viral DNA replication but also causes nuclear DNA damage during S-phase, followed by apoptosis. Our finding that GCV can trigger cellular senescence or cell death (in dividing cells), and can trigger cell death in non-dividing cells via mtDNA damage, explains some of the clinical efficacy of these compounds. Because senescent cells can be targeted by the immune system in vivo, inducing senescence in virally infected cells could accelerate their clearance.\(^\text{19–23}\) Alternatively, the senescence induced by GCV can be converted to cell death due to inhibition of mtDNA synthesis, leading to the further elimination of infected cells. Interestingly, the HSVtk-GCV combination is currently being tested in combination with herpes virus-derived oncolytic viruses to enhance tumor cell death in targeted cancer therapies.\(^\text{29–31}\) Our findings that GCV induces cell death and senescence even in cancer cells could explain the enhanced tumor cell elimination, possibly through an enhanced immune response against senescent cancer cells.

Senescent cells accumulate with age and are thought to cause or contribute to a variety of age-associated diseases.\(^\text{13–17,24,44}\) One striking characteristic of senescent cells is the secretion of inflammatory cytokines, proteases and other molecules that can alter the tissue microenvironment.\(^\text{18,33,45–47}\) We and others have shown that these secreted factors can disrupt normal tissue structure and function, induce malignant phenotypes in pre-malignant and...
non-aggressive cells and stimulate malignant tumorigenesis in vivo through inflammation and vascularization.\(^{13,14,18,45,46}\) Senescent cells are resistant to apoptosis, and, as the persistent presence of these cells can be harmful, it is important to find ways to eliminate them, preferably by causing apoptotic cell death, which occurs with minimal inflammation.

HSV\(\text{tk}\) and GCV have been used to induce apoptotic cell death in cell culture or tissues.\(^{35,41,49,50}\) We show that cells harboring HSV\(\text{tk}\) additionally undergo senescence in response to low GCV doses, whereas higher doses cause senescent cells to die by apoptosis owing to mtDNA damage. Because senescent cells increase their mitochondrial mass,\(^{38}\) they may be even more sensitive to GCV-mediated mitochondrial toxicity than non-senescent cells.\(^{40,41}\) Of note, as apoptosis was shown to be induced by persistent single-strand DNA breaks in the mitochondrial genome,\(^{51}\) the same mechanism could explain the effects of GCV we observed in senescent cells.

In addition to enhancing the potential effects of HSV\(\text{tk}\)-GCV based therapies, the fact that GCV can kill non-dividing senescent cells could be used to selectively eliminate senescent HSV\(\text{tk}\)-harboring cells in vivo—for example, if HSV\(\text{tk}\) were driven by a senescence-sensitive promoter, as recently described for another inducible killing strategy.\(^{24}\) Additionally, the multifunctional 3MR protein has the added advantage of providing imaging by bioluminescence and fluorescence.\(^{36,52,53}\)

A genetically engineered mouse model was recently developed to kill senescent cells in vivo using a drug that causes caspase activation.\(^{24}\) This study was pivotal in implicating a role for senescent cells in certain degenerative pathologies associated with aging. Here, our findings suggest killing senescent cells might also retard cancer progression. Using the HSV\(\text{tk}\)-GCV system described here, we are in the process of testing the idea that 3MR transgenic mouse models might allow tracking and eliminating senescent cells in vivo.

Materials and Methods

Cell culture. Human diploid HCA2 (foreskin) fibroblasts and human breast carcinoma cells (MDA-MB-231-D3H2LN Bioware expressing firefly luciferase;
PerkinElmer, Waltham, MA, USA) were cultured in Dulbecco’s modified Eagle’s media plus 10% fetal bovine serum and 100 U/ml streptomycin and penicillin.

Antibodies and reagents. Antibodies against 53BP1 (1 : 2000) and cytochrome c (1 : 800) were from Bethyl (Montgomery, TX, USA; BL-182) and PharMingen (No. 556432), respectively. The secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG (No. A11008, 1:750; Invitrogen, Carlsbad, CA, USA). Mouse monoclonal antibody against rLUC (1 : 200) was from Chemicon (Millipore, Billerica, MA, USA; MAB4400), and ganciclovir (GCV) was from Sigma (St Louis, MO, USA). Radio-labeled GCV was from Moravek Biochemicals Inc. (Brea, CA, USA).

Constructs. Stable expression of 3MR was achieved using lentiviruses generated as described.54 We cloned the 3MR sequence into a Gateway entry vector and transferred it to a lentiviral destination vector (670–1 puromycin resistance) under the control of the CMV/TO promoter.55

Irradiation and SA-gal staining. To induce senescence, cells were cultured to confluence, X-irradiated (10 Gy), allowed to recover overnight and re-seeded; 10 days later, cells displayed the enlarged senescent morphology and expressed SA-gal, as described.13 For SA-gal staining in tissues, 21 days following tumor inoculation, mice were euthanized and tumors collected; embedded in OCT, flash frozen in liquid nitrogen and stored at −80 °C. Embedded tumors were cryosectioned (10 μm), mounted on slides and stained for SA-gal using fast red as a counterstain.

Immunofluorescence. Cells were cultured in four- or eight-well chamber slides (Nunc, Penfield, NY, USA), fixed in 4% formaldehyde (Sigma) for 10 min at 4 °C and permeabilized in PBS-0.5% Triton for 10 min at 4 °C. Slides were blocked for 30 min in blocking buffer (1% IgG-free BSA, 4% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS) and incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature or overnight at 4 °C. Cells were washed, incubated with secondary antibodies for 30 min at room temperature, washed and mounted with slow-fade gold (Molecular Probes, Invitrogen) or Vectashield containing DAPI (Vector Labs, Burlingame, CA, USA). Images were acquired using an Olympus BX20 fluorescence microscope with spotfire software (Diagnetics Instruments, Sterling Heights, MI, USA) and processed with Photoshop CS (Adobe, San Jose, CA, USA).

DNA synthesis detection. Cells in chamber slides were pulsed with the modified thymidine analogue EdU for 24 h before fixation and permeabilization, as described above. Cells were processed as per the manufacturer’s protocol to detect the incorporation of EdU into DNA using Click-IT chemistry (Invitrogen C10340). Slides were washed with PBS and mounted with Vectashield containing DAPI (Vector Labs) and analyzed for immunofluorescence.

Flow cytometry analysis for apoptosis detection. Live trypsinized cells and their supernatant were collected, centrifuged, rinsed with PBS and processed according to the manufacturer’s apoptosis detection kit protocol, except that propidium iodine was replaced by DAPI at a concentration of 800 ng/ml. Slides were blocked for 20 min in blocking buffer (1% BSA, 0.1% NaN3, 200 μg/ml bovine serum albumin, 2 mg/ml heparin), washed with PBS, and covered with DAPI (1 mg/ml). Coverslips were mounted on slides using SlowFade Gold mounting medium and analyzed by a Fluoview FV1000 confocal microscope (Olympus). Statistical analysis was performed using Graphpad Prism software (GraphPad Software). Significance was assessed using Student’s t-test (2-tailed) with a significance level of 0.05.

Measurement of GCV incorporation and mtDNA damage. HCA2 and HCA2-3MR fibroblasts were X-irradiated (10 Gy) to induce senescence. Ten days later, cells were given 0.1 μM tritiated GCV (1H-GCV; 3.9 Ci/mmol) (Moravek Biochemicals) for 48 h, and washed. Mitochondria and mtDNA were purified using Mitochondria Isolation (Pierce, Rockford, IL, USA) and Mitochondrial DNA Isolation kits (Aaboom, San Francisco, CA, USA). Scintillation fluid was mixed with 100 ng of mtDNA (evaluated by densitometry on agarose gels) and radioactivity was quantified. For DNA damage analysis, cells were treated similarly but incubated with 10 μg/ml cold GCV for 48 h and loaded onto 0.5% agarose gels. DNA was stained with SYBR Gold (Invitrogen) and detection performed using a Typhoon apparatus (GE Healthcare Biosciences, Pittsburgh, PA, USA).

Tumor growth assays. Tumor growth was followed as described.13,45,56 Briefly, we injected into the mammary fat pads of 5-week-old nu/nu mice a 100-μl suspension of 106 MDA-MB231-D3H2LN (control) or MDA-MB231-D3H2LN-3MR cells and monitored tumor growth by firefly luminescence using a IVIS Spectrum II Biophotonic imager (Xenogen, PerkinElmer) and caliper measurements over a 21-day period. The clearance of HCA2-3MR primary fibroblasts after injection of 5 × 103 cells was performed similarly, except luminescence from rLUC in 3MR was detected over 10 days. Mice were treated with 25 mg/kg of GCV by daily intraperitoneal injections over 5 days.

Statistical analysis. Error bars on all graphs are the S.D. of three independent measurements. Statistical analyses were performed using Graphpad Prism software (GraphPad, La Jolla, CA, USA).

Conflict of Interest

The authors declare no conflict of interest.

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