Cellular senescence: from growth arrest to immunogenic conversion

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Abstract Cellular senescence was first reported in human fibroblasts as a state of stable in vitro growth arrest following extended culture. Since that initial observation, a variety of other phenotypic characteristics have been shown to co-associate with irreversible cell cycle exit in senescent fibroblasts. These include (1) a pro-inflammatory secretory response, (2) the up-regulation of immune ligands, (3) altered responses to apoptotic stimuli and (4) promiscuous gene expression (stochastic activation of genes possibly as a result of chromatin remodeling). Many features associated with senescent fibroblasts appear to promote conversion to an immunogenic phenotype that facilitates self-elimination by the immune system. Pro-inflammatory cytokines can attract and activate immune cells, the presentation of membrane bound immune ligands allows for specific recognition and promiscuous gene expression may function to generate an array of tissue restricted proteins that could subsequently be processed into peptides for presentation via MHC molecules. However, the phenotypes of senescent cells from different tissues and species are often assumed to be broadly similar to those seen in senescent human fibroblasts, but the data show a more complex picture in which the growth arrest

mechanism, tissue of origin and species can all radically modulate this basic pattern. Furthermore, well-established triggers of cell senescence are often associated with a DNA damage response (DDR), but this may not be a universal feature of senescent cells. As such, we discuss the role of DNA damage in regulating an immunogenic response in senescent cells, in addition to discussing less established “atypical” senescent states that may occur independent of DNA damage.

Keywords Immunogenic · Senescence · Immune surveillance · Apoptosis resistance · Secretome · NKG2D

The senescent cell and its phenotype

Hayflick and Moorhead (1961) were the first researchers to exclude poor tissue culture techniques or differential nutritional requirements as explanations for the experimental observation that multiple strains of normal human fibroblasts failed to multiply indefinitely in vitro. The demonstration that this was a cell-intrinsic phenomenon, coupled with the observation that strains of embryonic fibroblasts grew better than those derived from adults, led the authors to coin the term ‘cell senescence’ and propose that this failure of proliferation could be related to organismal ageing (Hayflick 1965). The demonstration by Macieira-Coelho et al. (1966) that senescent cells were viable (as measured by ³H-uridine uptake) combined with both the observation of an inverse relationship in vitro between donor age and growth capacity in normal human fibroblast cultures

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(Goldstein 1969; Martin et al. 1970) and the discovery that cells from patients with the accelerated ageing disease Werner's syndrome showed premature senescence (Epstein et al. 1966) laid the basis of a conceptual framework for the study of the relationship between cell senescence and ageing which continues to this day.

Permanent exit from the cell cycle still remains the characteristic most closely associated with "senescent cells". This is perhaps because the phenotype investigated in these early studies was both striking and readily observed (as well as being most relevant to researchers primarily interested in cancer). However, by the late 1980s, it was becoming clear that permanent cell cycle arrest was not the only feature that distinguished a senescent cell from its growth competent counterparts. For example, West et al. (1989) demonstrated that senescent fibroblasts constitutively produce high levels of pro-collagenase (and low levels of collagen) compared to their growing counterparts. As such, senescent cells slowly started to be considered as phenotypically 'active players' in tissue, rather than simply cells in a passive state of cell cycle arrest.

Alterations in the spectrum of growth factors and matrix remodeling enzymes secreted by senescent fibroblasts were subsequently reported (reviewed in Faragher and Kipling 1997), but, retrospectively, these findings must be judged incremental compared to the comparative microarray work of Shelton et al. (1999) which demonstrated that senescent fibroblasts expressed a wide range of inflammatory associated genes similar to an early remodeling phase of wound repair (including ICAM-1, MCP-1, Gro- α IL-1 and IL-1 β). Nearly a decade later, a landmark paper by Coppé et al. (2008) extended these observations to the secretome through the use of antibody arrays. They observed a conserved pattern of secreted molecules between five senescent human fibroblast strains regardless of whether senescence was induced by telomere attrition or global genomic damage. This phenotype included inflammatory cytokines (e.g. IL-6, IL-8 and MCP-2), growth factors (e.g. Gro and scatter factor) and cell surface molecules (e.g. ICAM and uPAR/CD87).

The reason why senescent fibroblasts adopted this secretory phenotype was initially unclear. A growing body of evidence now suggests that the key feature of the senescent state *in vivo* may not be permanent cell cycle arrest *per se* but rather a process we term "immunogenic conversion". We define this as a shift from a passive cellular phenotype in terms of immune

surveillance to one that actively promotes self-elimination by the immune system. In this model, the secretory phenotype of senescent cells serves to attract immune cells (Sagiv and Krizhanovsky 2013), which then recognize their target via the up-regulated ligands found on their surface (Krizhanovsky et al. 2008; Kim et al. 2008; Soriani et al. 2009; Chuprin et al. 2013).

Transition to a senescent state, rather than an apoptotic one, may be optimal in certain physiological contexts since this can preserve tissue integrity that might otherwise be compromised through cell loss (Burton and Krizhanovsky 2014). However, the length of time within the tissue is clearly a major factor in determining whether the effect of entry into senescence will be beneficial or deleterious to the organism. The appearance of senescent cells in the short-term probably facilitates important physiological functions such as tumour suppression (Braig et al. 2005; Chen et al. 2005; Michaloglou et al. 2005; Collado et al. 2005), wound healing (Krizhanovsky et al. 2008; Jun et al. 2010; Fitzner et al. 2012; Kim et al. 2013; Demaria et al. 2014) and possibly placental development (Chuprin et al. 2013; Rajagopalan and Long 2012; Zhang et al. 2014). However, senescent cells are known to accumulate *in vivo*, possibly as a result of impaired immune clearance by an ageing immune system (Burton 2009). As such, age-related long-term persistence of senescent cells in tissues can potentially promote pro-inflammatory pathological conditions, mediated partly by the same secretory response that initially aided in immunosurveillance of senescent cells (Ovadya and Krizhanovsky 2014).

Triggers of 'senescence' and the senescent phenotype

A wide range of triggers of cellular senescence is now known. The most famous of these is probably progressive telomere attrition as a result of sustained cell cycle traverse (replicative senescence, RS) (Herbig et al. 2004). This is the mechanism underpinning Hayflick's observation of senescence in human fibroblasts *in vitro*. A range of other human cell types also use this mechanism. However, it is important to recognize that not all human cell types use progressive telomere attrition as a primary arrest mechanism (Kiyono et al. 1998; Evans et al. 2003) and that cells from some other species (e.g. mice) do not use it at all (Smith and Kipling 2004; Itahana et al. 2004). Oncogene activation (oncogene-

induced senescence, OIS) (Serrano et al. 1997; Zhu et al. 1998), elevated reactive oxygen species (stress-induced premature senescence, SIPS) (Toussaint et al. 2000) and cell-cell fusion (Chuprin et al. 2013) all trigger entry into a senescent state.

With so many potential triggering mechanisms available across many different cell types in multiple species, some sub-division of the semantic domain currently covered by the term “cell senescence” is probably utile. On a conceptual level, this would allow practical experiments to be proposed which shed light on the roles that these various types of growth-arrested cells may play in vivo. Accordingly, we propose that immunogenic conversion represents a key phenotypic distinction between the different types of permanent growth arrest that are all currently (and indifferently) labeled “senescent”. Put simply, senescent cells that have undergone immunogenic conversion have the potential both to be actively cleared in tissue (short-term immune response) and to be actively deleterious (long-term inflammatory response). Those that have arrested, but have not converted are probably less likely to cause damaging changes to tissues in vivo. However, the possibility exists that these cells would still exhibit abnormal protein expression that could impact their microenvironment, but further evaluation is required. In addition, if present in large enough numbers, growth arrested, non-immunogenic senescent cells could persist and subsequently reduce the regenerative potential of tissues, more so if these cells are stem cells.

Many triggers of cell senescence result in DNA damage in cells in vitro (Di Micco et al. 2006; Hewitt et al. 2012; Chuprin et al. 2013) and in vivo (Herbig et al. 2006; Wang et al. 2009; Suram et al. 2012). This observation gives rise to the important conceptual question of whether the DNA damage response (DDR) is an absolute requirement for immunogenic conversion. Therefore, we discuss the role of DNA damage in regulating cell cycle arrest, the secretory response and expression of immune ligands, as well as presenting a rationale for a role of DNA damage in initiating pro-survival responses and promiscuous gene expression (Fig. 1).

Cell cycle arrest

“DNA damage” is a general term subsuming various kinds of DNA lesions that can consequently result in

different biological responses depending upon the biological context (e.g. proliferating/non-proliferating cells) (Vermeij et al. 2014). DNA lesions include, but are not limited to, single strand breaks (SSBs), double strand breaks (DSBs), interstrand cross-links (ICL), mismatch and abasic (Vermeij et al. 2014). The majority of the work on cellular senescence is primarily related with double strand breaks (DSBs), as this form of DNA damage is irreparable, persistent and evokes a DDR (Chen et al. 2007).

In response to DNA damage, proliferating cells enter a state of cell cycle arrest that allows them to repair the damage. If it is repaired, the cell can re-enter the cell cycle. In contrast, cellular senescence appears to be initiated when a persistent DDR occurs (Rodier et al. 2009; Fumagalli et al. 2014), such as that resulting from dysfunctional telomeres or activated oncogenes (d’Adda di Fagagna 2008). Upon sensing DNA damage, the ataxia telangiectasia mutated (ATM), ataxia telangiectasia and RAD3-related (ATR) gene products inhibit cell cycle progression by promoting p53 accumulation, which subsequently regulates a number of target genes, including the cyclin-dependent kinase inhibitor (CDKi), p21/Cip (Herbig et al. 2004). p21 binds to and inhibits the activity of cyclin-dependent kinase (CDK)2 and CDK4 complexes leading to dephosphorylation of retinoblastoma (RB) and consequently preventing the release and activation of E2F-dependent gene expression. ATM and ATR appear to respond to different types of DNA damage (Smith et al. 2010). ATM is primarily a mediator of the response to double strand breaks (DSBs) whilst ATR appears to function in response to persistent single-stranded DNA damage. Experimental activation of ATR has been shown to induce cell senescence in the absence of DNA damage (Toledo et al. 2008), demonstrating that it is activation of the DDR and not the presence of DNA damage per se that is important. Whether activation of a DDR in the absence of DNA damage can occur in normal physiological settings has yet to be determined, but could potentially occur. Irreparable persistent DNA damage signaling subsequently leads to the up-regulation of p16(INK4a), which seems to function in the long-term maintenance of cell cycle arrest (Robles and Adami 1998). It appears that if quiescent cells acquire DNA damage, cell cycle traverse is required to initiate a full DDR resulting in their conversion from quiescence to senescence (Sousa-Victor et al. 2014). Induction of cell senescence by oncogene activation in vivo has also been

Fig. 1 Schematic representation showing the role and potential role of the DNA damage response in mediating multiple features of senescent fibroblasts, including cell cycle arrest, the secretory

response, the up-regulation of immune ligands, pro-survival response and promiscuous gene expression

reported to require cell cycle progression (Di Micco et al. 2006).

When human fibroblasts enter senescence, there appears to be a number of positive feedback loops designed to maintain the proliferative arrest through sustained activation of the DDR. For example, long-term activation of p21 leads to mitochondria dysfunction and subsequently elevated ROS (Passos et al. 2010). In the short-term, the elevation in ROS likely functions as secondary messengers that induce pro-inflammatory innate immune responses (Palmai-Pallag and Bachrati 2014). However, long-term elevation in ROS may continuously generate damage to DNA, thereby maintaining a persistent DDR. Effects of this type are not confined to fibroblasts. In human thyrocytes, NADPH oxidase Nox4 has been shown to maintain a DDR during OIS through the generation of ROS (Weyemi et al. 2012). It has also been shown in U2OS cells and BJ fibroblasts that induction of ROS and DDR via Nox4 drives induction of senescence in growth-competent

nearby cells (the so-called “bystander effect”), a process regulated by secretory factors such as IL-1 (Weyemi et al. 2012; Hubackova et al. 2012). The secretion of other cytokines (e.g. IL-6 and IL-8) by senescent cells has also been shown to be important for maintaining the senescent state via autocrine feedback loops that generate DNA damage (Acosta et al. 2008; Kuilman 2008). The presence of microRNA feedback loops has also been reported to play a role in activating p16 in senescent cells (Overhoff et al. 2013).

The relationship between cell senescence and the secretory phenotype

When embryonic lung, neonatal foreskin, or adult breast fibroblasts become senescent as a result of DNA damage, they up-regulate and secrete common soluble factors consisting of pro-inflammatory cytokines, growth factors and proteases (Shelton et al. 1999; Coppé et al.

2008). The same response is also observed in other cell types such as senescent human vascular smooth muscle cells (Burton et al. 2009). Fibroblasts that have undergone permanent cell cycle arrest by experimentally over expressing p16INK4a do not seem to display a secretory phenotype until inflicted with DNA damage, thus demonstrating the importance of a DDR in generating this response (Coppé et al. 2011). Therefore, the secretory response of senescent cells appears to be uncoupled from cell cycle arrest but dependent upon a common effector such as DNA damage.

The senescent secretome, regulated by pathways associated with IL1beta (Acosta et al. 2013), HMGB1 (Davalos et al. 2013), p38 (Freund et al. 2011) and NFkB (Chien et al. 2011) appears to be similar to a wound healing response, likely facilitating in immunosurveillance of senescent cells. Many of the proteins commonly secreted by senescent fibroblasts are capable of activating and attracting immune cells such as neutrophils, monocytes/macrophages, T-cells and natural killer (NK) cells (reviewed in Sagiv and Krizhanovsky 2013). Xue et al. demonstrated that the induction of senescence by reactivation of p53 in p53 null tumours resulted in elevated expression of chemokines and adhesion molecules that promoted immune clearance by neutrophils and NK cells, thereby limiting tumour growth (Xue et al. 2007). In addition to preventing tumour development, the elimination of senescent activated hepatic stellate cells by NK cells during acute liver damage was shown to limit the fibrogenic response (Krizhanovsky et al. 2008). Sagiv et al. demonstrated that NK cell mediated cytotoxicity of senescent cells is preferentially through the granule exocytosis pathway rather than through death-receptor mediated apoptosis (Sagiv et al. 2013).

In addition to NK cells, T-cells and macrophages also appear to play a role in immunosurveillance of senescent cells. It was shown that oncogene-induced senescent hepatocytes within the livers of mice secreted various cytokines/chemokines and were consequently subjected to immune clearance by a T-cell-mediated response that also required the function of monocytes/macrophages (Kang et al. 2011). Impaired immune surveillance resulted in the development of murine hepatocellular carcinomas. In another study, senescent hepatic stellate cells secreted factors that favoured macrophage polarization towards a tumour-inhibiting M1-state capable of targeting senescent cells (Lujambio et al. 2013).

These studies highlight the importance of immune-mediated clearance of senescent cells for maintaining normal tissue homeostasis. However, this process may be disrupted in pathological conditions. For example, *Pten*-loss-induced senescence in prostate tumours has been shown to instead generate an immunosuppressive secretome (Toso et al. 2014), whilst in other contexts, immune cells have been shown to induce cell senescence in tumour cells (Rakhra et al. 2010; Reimann et al. 2010; Braumuller et al. 2013), a process that likely functions in preventing tumour growth. Therefore, it is important to evaluate whether the senescent secretome in unexplored cell models, particularly in pathological conditions, promotes or inhibits immune surveillance.

Since the senescent secretome appears to be part of a DDR, it is doubtful that it is specific to senescent cells and likely occurs in other cell contexts associated with DNA damage. For example, DNA damage and high ROS are present in more than 40 % of post-mitotic cortical, hippocampal and peripheral neurons in the myenteric plexus of old C57Bl/6 mice (Jurk et al. 2012) alongside a phenotype and secretory response (including elevated IL-6 production) similar to that of a senescent cell. The effect of this on immune cells entering the CNS (e.g. via the choroid plexus) is unknown and potentially of considerable interest. Similar to senescent cells, cancer cells can also display a DDR with a secretory response (Gilbert and Hemann 2010). This at first sight seems paradoxical, but if tumour cells were once senescent but escaped the growth arrest, then there is nothing intrinsically implausible in this. This process would generate proliferating cells with a partial senescent phenotype. Similarly, cells that instead bypass the senescence programme may potentially induce a secretory response as a result of genomic instability due to unregulated cellular proliferation. DNA damaged-induced pro-inflammatory responses have also been reported in other systems mediated via ATM (Karakasilioti et al. 2013; Takacova et al. 2012). Thus, although a secretory phenotype is associated with cell senescence, it does not seem to be directly driven by growth arrest and could occur in other biological settings which activate a DDR.

There is some evidence from the literature that the relationship between DNA damage, senescence and the secretory phenotype is cell type, species and environment specific. Thus, mouse embryonic fibroblasts cultured under conditions of supraphysiological oxygen (20 %) do not show a secretory response but do show

one similar to that seen in human fibroblasts if the partial oxygen pressure is reduced to the more 'physiological' level of 3 % (Coppé et al. 2010). Microarray analysis of replicative senescent fibroblastoid keratocytes (which are known to enter senescence in response to telomere attrition and thus have an active DDR) did not show transcriptional up-regulation of senescence associated inflammatory factors but rather a broader 'dysdifferentiation' (Kipling et al. 2009). The fact that the eye is an immune-privileged site (and thus the keratocytes in question cannot act as targets for clearance) may be relevant in this latter case. Whilst OIS is often associated with an elevation in ROS and DNA damage, the activation of oncogenes such as Ras could potentially activate pathways involved in a secretory response, such as p38, independent of DNA damage (Freund et al. 2011). In addition, the activation of different oncogenes may evoke an oncogene-specific response (Maya-Mendoza et al. 2014) that may be reflected in variation in the secretory response.

Overall, it is probably unwise to assume that the secretory phenotype is (i) invariably present in senescent cells, (ii) present if conditions favouring DNA damage are in place or (iii) absent if they are not. As such, a case-by-case assessment of the secretory phenotype is recommended.

The relationship between cell senescence and immune ligand expression

In addition to secreting soluble factors for the attraction of immune cells, senescent cells can also become immunogenic through the up-regulation of ligands that can specifically be recognized by immune cells. Whilst research into the recognition and interaction of immune cells with senescent cells is at its infancy, a number of studies have reported the up-regulation of the natural killer group 2D (NKG2D) ligands in senescent cells that can be recognized by receptors on natural killer (NK) cells and CD8⁺ T-cells. Since NKG2D ligands are not widely expressed on healthy cells, this would allow for specific recognition, interaction and elimination of senescent cells by immune cells. As with the senescent secretome, this response is likely not exclusive to cell senescence as the same mechanism functions in immunosurveillance of tumour cells (López-Soto et al. 2014). The human NKG2D ligands primarily consist of MICA, MICB, ULBP1, ULBP2, ULBP3, ULBP4,

ULBP5 and ULBP6. The transcriptional up-regulation of MICA and ULBP2 during cell senescence have been reported in senescent-activated hepatic stellate cells, replicative senescent fibroblasts and HUVECs, etoposide-induced senescent fibroblasts, fusion-induced senescent fibroblasts and chemotherapy-induced senescent multiple myeloma cells (Krizhanovsky et al. 2008; Kim et al. 2008; Chuprin et al. 2013; Soriani et al. 2009; Lackner et al. 2014). In addition to MICA and ULBP2, microarray analysis of replicative senescent fibroblasts demonstrated an increase in the expression of ULBP1 (2.75-fold) compared to growing cells, in addition to the up-regulation of HLA-E (2-fold) (Lackner et al. 2014). HLA-E is a non-classical MHC class I molecule that plays a role in cell recognition by NK cells. However, replicative senescent vascular smooth muscle cells do not appear to up-regulate MICA, ULBP2 or ULBP1, at least not greater than twofold as assessed by microarray analysis (Burton et al. 2009). Therefore, it should not be assumed that all senescent cell types up-regulate NKG2D ligands, and this should be evaluated in underexplored senescent cell types. Mechanisms involved in the interaction of senescent cells with T-cells is less understood, but it appears that major histocompatibility complex class II (MHCII) expression is required for killing of pre-malignant senescent hepatocytes by T-cells (Kang et al. 2011). Mice with liver specific MHCII deficiency resulted in impaired immunosurveillance of senescent cells.

At the mechanistic level, little is currently known about the regulation of NKG2D ligand expression in senescent cells. Nonetheless, some extrapolation from others models is possible. For example, MICA and MICB have been reported to be regulated by endogenous miRNAs in tumours and as a result of infection with cytomegalovirus (Stern-Ginossar et al. 2008). Since miRNAs appear to play a role in regulating cellular senescence (Feliciano et al. 2011; Liu et al. 2012; Benhamed et al. 2012) and their expression is altered in response to DNA damage (Dolezalova et al. 2012; Wang and Taniguchi 2013), it is possible that changes in miRNA expression also regulate the expression of immune ligands in senescent cells.

Soriani et al. demonstrated that the up-regulation of MICA in senescent multiple myeloma cells was dependent upon the DDR (Soriani et al. 2009). In other systems, NKG2D ligands have also been shown to be up-regulated in response to DNA damage and Ras

activation via ATM and ATR (Gasser et al. 2005; Cerboni et al. 2014). Inhibition of the ATM or ATR pathways prevented the up-regulation of immune ligands.

It is also possible that the up-regulation of immune ligands on senescent cells is mediated via the secretory response. In addition to activating and attracting immune cells, the senescent secretome may serve to up-regulate immune ligands in an autocrine or paracrine manner. It has been shown for example, that TNF α can up-regulate MICA on human endothelial cells and that the addition of exogenous MICA seems to induce senescence in HUVECs (Lin et al. 2012), but the extent to which this occurs under more physiologically reflective situations remains unclear.

Immune ligands can also be up-regulated in response to various other forms of cell stress such as heat shock, metabolic stress and endoplasmic reticulum (ER) stress (Cerwenka 2009; Valés-Gómez et al. 2008). Thus, as with the secretory response, mechanisms exist that can up-regulate immune ligands independent of DNA damage. Given that this is an important aspect of senescent cell clearance and the number of cell types in which the up-regulation of immune ligands has been shown is limited, a more detailed study of this aspect of immunogenic conversion seems warranted.

Whilst senescent cells are likely eliminated by the immune system during normal physiological processes, it has been speculated that the accumulation of senescent cells with age could be due to inefficient elimination by an ageing immune system (Burton 2009). In fact, immune cells may themselves undergo cellular senescence, a process that requires further investigations (Effros et al. 2005; Rajagopalan et al. 2012). As such, induction of cell senescence in immune cells may represent one aspect of immunosenescence, the gradual deterioration of the immune system, which consequently leads to impaired immunosurveillance of non-immune senescent cells. It can be speculated that impaired immunosurveillance may result from altered expression of surface receptors on immune cells that impair recognition and interaction with target senescent cells (and cancer cells). In addition, it is possible that aged or senescent immune cells do not respond as efficiently to chemoattractants secreted by senescent cells. In order to understand the mechanisms associated with age-related changes resulting in impaired immunosurveillance of senescent cells, we must first fully understand the normal processes governing

immune clearance of senescent cells. However, evaluating the hypothesis that aged or senescent immune cells display a reduced capacity to target senescent cells and the physiological impact of this decline can still be assessed. If this were indeed found to be the case, the rejuvenation of an ageing immune system would represent an attractive approach for promoting health span.

The relationship between senescence and ‘apoptosis resistance’

Senescent cells are frequently referred to as ‘apoptosis resistant’. This apparent resistance to an apoptotic stimulus *in vitro* was originally reported by Wang (1995) who observed that late passage (58 population doubling) WI38 fibroblasts were resistant to death caused by serum withdrawal compared to WI38 cultures at less than 15 or approximately 38 population doublings. All of these human cell populations were dramatically more resistant to death by growth factor deprivation than Swiss 3T3 fibroblasts. This death-resistant phenotype was linked to maintenance of Bcl2 protein levels in senescent WI38 cells. Subsequent studies extended the resistance phenotype to treatment with both UV light (120 mJ) and staurosporine (35 nM) and linked it to reduced expression of caspase 3 (Marcotte et al. 2004). Subsequent work (Ryu et al. 2007) using human dermal fibroblasts confirmed resistance to staurosporine-induced cell death and demonstrated significant resistance to thapsigargin (up to 700 nM). The enhanced survival of senescent dermal fibroblasts under these conditions was attributed to a failure to down-regulate Bcl2 under conditions of cellular stress.

It has been proposed that resistance to apoptotic cell death is a feature of the senescent phenotype that may promote their persistence *in vivo*, thereby favouring immune clearance over cell death. However, key questions around this phenotypic aspect remain and may be summarized as (i) what are the primary molecular players driving apoptosis resistance in senescent human dermal and lung fibroblasts? (ii) is this phenomenon a general one across tissues and between species?

It is possible that the pro-survival response observed in fibroblasts normally facilitates DNA repair, but is maintained when persistent DNA damage activates the senescent programme. For example, when low levels of DSBs are present, ATM and ATR can result in ERK/NF κ B pro-survival signaling (Khalil et al. 2011;

Hawkins et al. 2011; Janssens and Tschopp 2006) that has been associated with the induction of senescent cells by various triggers. Paradoxically, ATM-deficient human fibroblasts are significantly more resistant to cell death triggered by exposure to doxorubicin or low-dose ionizing radiation than wild-type controls (Park et al. 2013). However, the population doublings levels of the wild-type and mutant cultures were not reported. If significantly different, this has the potential to confound studies of this type (since normal fibroblast cultures are mixtures of senescent and proliferating cells, the proportions of which alter as the culture is passaged).

In addition to activating cell cycle arrest in response to DNA damage, the p53/p21 pathway can also initiate a pro-survival response. In some studies, p21 has been shown to play a role in cell survival through its cytoplasmic localization, rather than its nuclear localization associated with cell cycle arrest (Gartel and Tyner 2002; Piccolo and Crispi 2012; Kreis et al. 2014). Interestingly, p21 has been reported to be a negative regulator of p53-mediated apoptosis (Gartel and Tyner 2002), a known response reported in senescent fibroblasts (Seluanov et al. 2001). p21 has also been reported to promote cell survival in response to oxidative stress by integrating the DDR with endoplasmic reticulum (ER) stress signaling (Vitiello et al. 2009). However, the up-regulation of p21 may also be required for cells to enter and maintain quiescence (Perucca et al. 2009), suggesting a pro-survival response may occur independent of DNA damage, but dependent upon growth state.

Autophagy is another feature of senescent cells which can also be initiated by DNA damage and promote cell survival (Rodriguez-Rocha et al. 2011; Singh et al. 2012). Autophagy promotes cell survival by the degradation of damaged cellular components (Codogno and Meijer 2005), probably as a result of elevated ROS (Scherz-Shouval and Elazar 2011) in the case of cell senescence. Interestingly, there is crosstalk between autophagy and apoptosis pathways (Zhou et al. 2011; Xu et al. 2013; Lindqvist and Vaux 2014), with particular emphasis on the anti-apoptotic Bcl2 protein family.

It has long been recognized that cytokines and their binding proteins can act to modulate cell survival (Lotem and Sachs 1999). Given the altered secretory phenotype of some senescent cells, it would be unsurprising if this did not contribute to altered death dynamics, but the mechanisms by which this could occur are potentially highly complex. For example, interleukin-6 (secreted by senescent cells) has been shown to promote

cell survival in transformed cells (Biroccio et al. 2013), and its secretion by cancer-associated fibroblasts protects luminal breast cancer cells from tamoxifen treatment (Sun et al. 2014). Whilst inhibition of insulin-like growth factor-1 (IGF-1) has been shown to induce apoptosis in senescent fibroblasts (Luo et al. 2014), the alteration of IGF-1 binding proteins are just as likely to influence cell survival. For example, insulin-like growth factor binding protein 3 (IGFBP-3) is both transcriptionally up-regulated and secreted in elevated amounts by senescent human fibroblasts (Hampel et al. 2005). IGFBP-3 triggers enhance apoptotic cell death in tumour cells when internalized and translocated to the nucleus, where it targets intracellular regulators of apoptosis (Hampel et al. 2005). Endocytotic uptake of IGFBP-3 in senescent human fibroblasts did not occur. This has the potential to render them apoptosis resistant and capable of promoting apoptosis in cells nearby. It could be speculated that in a microenvironment characterized by high cell turnover, both senescent and pre-cancerous cells could be in close proximity. Elevated local IGFBP-3 generated by senescent cells could thus act as a paracrine tumour suppression mechanism. This idea remains untested.

It seems doubtful that global apoptosis resistance is a general feature of senescent cells. For example, early work by one of us (RGAF) failed to show any elevation in spontaneous apoptosis rates in HUVECs cultured to senescence (although baseline apoptosis rates as measured by TUNEL were significantly higher than those seen in fibroblasts) (Kalashnik et al. 2000). Later studies (Hoffmann et al. 2001) demonstrated that late passage HUVECs were more sensitive to apoptosis induced by oxidized LDL or TNF α compared to early passage cells. Jeon and Boo (2013) have recently shown that up-regulation of the Fas receptor at both the mRNA and protein level in senescent HUVECs probably underlies their enhanced potential to undergo programmed cell death. Perhaps most compellingly, Hampel et al. (2004) demonstrated in parallel culture experiments that whilst senescent human dermal fibroblasts were more resistant to cell death induced by exposure to ceramide than early passage cells, senescent HUVECs were significantly more apoptosis prone.

It is interesting that minimal changes in baseline apoptosis rates could be detected in senescent HUVEC populations despite their increased sensitivity to Fas or ceramide-induced killing. However, Wang et al. (2004) reported an analogous phenomenon in senescent human

keratinocytes. This study demonstrated that spontaneous apoptosis rates did not alter in cultures of senescent human keratinocytes (duplicating an earlier report by Norgaard et al. 1996). Nonetheless, levels of Fas and related apoptotic effectors (e.g. FLICE) increased whilst Bcl2 declined significantly (as measured by ELISA). The authors showed that antibody-mediated Fas activation or medium exhaustion increased the apoptotic fraction from 3–5 to 30 % in senescent keratinocytes, whilst leaving apoptosis levels unchanged in early passage cultures.

Interestingly, Crescenzi et al. (2011) have recently shown that induction of premature senescence in human cancer cell lines also induces Fas expression, and concomitant susceptibility to Fas-induced apoptosis. Fibroblasts rendered senescent by serial passage are also susceptible to Fas-mediated killing (Tepper et al. 2000). Thus, it is possible that at senescence, human cell types differ in their resistance to apoptosis induced by stressors, but show a common susceptibility to Fas/TNF α -mediated killing. If immunogenic conversion were a key hallmark of senescence, then this would seem plausible. It does however require significant additional experimental study.

As with the secretory response, it should not be assumed that an “apoptosis resistant” phenotype is conserved across species. For example, Mayogora et al. (2004) demonstrated that cultures of cardiac fibroblasts from Sprague Dawley rats were more resistant to apoptosis induced by serum withdrawal or staurosporine, than dermal fibroblast cultures initiated from the same animals. Dermal fibroblasts from this species apparently lacked Bcl2 protein as measured by Western blot (although it remained readily detectable in cardiac fibroblasts). This is a clear species difference and suggests that researchers working in other systems should not assume that the features observed in human cells are duplicated across the animal kingdom.

The senescent phenotype and promiscuous gene expression

Senescent cells are often associated with changes in gene expression that appear to occur independent of the regulated gene expression linked to aspects of the senescent phenotype such as cell cycle arrest, the secretory response and apoptosis resistance. This phenomenon has been termed promiscuous gene expression

(pGE) (Burton and Krizhanovsky 2014) and can be more specifically defined as gene expression that is uncoupled from tissue or developmental regulation.

pGE can be observed in microarray analysis by comparing the gene expression profiles of different senescent cell types and lines. Zhang et al. (2003) has demonstrated that the up-regulation of genes in senescent fibroblasts was associated with gene clustering (150 of the 376 gene up-regulated), whereas the down-regulation of genes (313) was not; 48.1 % of the up-regulated genes were designated as membrane-associated proteins, 10.5 % related to apoptosis and 15.8 % to transport, whereas 17.9 % of the down-regulated genes are involved in cell cycle regulation. Gene expression changes in senescent human mammary epithelial cells (HMECs) were shown to be drastically different than that of the fibroblasts, despite both undergoing senescence induced by telomere attrition. Only five genes up-regulated and seven genes down-regulated in HMECs showed similar regulation in fibroblasts. However, like senescent fibroblasts, HMECs also demonstrated gene clustering associated with up-regulated genes only. Zhang et al. postulated at the time that if senescence is a response to DNA damage, then the observed differences in gene expression between senescent fibroblasts and HMECs imply that the effects of DNA damage must vary according to cell type and line. This study also suggested that processes occurring during senescence may lead to localized alteration in chromatin and the consequent up-regulation of groups of genes within “opened” domains.

Shelton et al. (1999) also demonstrated that senescence-mediated gene expression between different cell lineages varies greatly. BJ fibroblasts, HUVECs and retinal pigment epithelial cells (RPE340) that underwent replicative senescence demonstrated substantial variation in gene expression. A genomic comparison of three different senescent fibroblasts strains also demonstrated significant differences in gene expression, but also shared trends were apparent. If indeed pGE is uncoupled from tissue or developmental regulation, then stochastic processes that alter chromatin structure could be at play and the different response between cell types and cell strains could reflect differences in cell-specific chromatin architecture important for cell-specific gene expression. Elevated levels of oxidative stress, a feature of senescent cells could be one such stochastic process.

Bahar et al. demonstrated that although gene expression levels varied amongst cardiomyocytes taken from

hearts of young mice, the heterogeneity is elevated with age (Bahar et al. 2006). This increased stochastic gene expression with age was suggested to be the result of genomic damage, as mouse embryonic fibroblasts treated with hydrogen peroxide in culture resulted in significant cell-cell variation in gene expression in conjunction with these cells showing morphological signs of cellular senescence (Bahar et al. 2006).

So how could DNA damage induced by oxidative stress result in stochastic changes in gene expression? When cells sustain DNA damage, chromatin undergoes remodeling to facilitate DNA repair (Price and D'Andrea 2013; House et al. 2014). This remodeling or “opening” of tightly packed DNA could allow transcription factors access to previously inaccessible genes. Therefore, persistent DNA damage and consequently continuous chromatin remodeling may facilitate pGE. Whilst the induction of DNA damage is likely a stochastic process, the sites of DNA damage may not be completely random, as certain areas of the genome may be more or less prone to genomic insults (Ma et al. 2012). The clustering phenomenon reported by Zhang et al. may be the result of these DNA damage prone sites (Zhang et al. 2003). If this were indeed the case, whilst there may be substantial differences in gene expression at a cell-cell comparison, an overall comparison between cell cultures would likely demonstrate consistent gene alterations resulting from an average expression of all cells within a culture.

In addition to oxidative stress, a number of other possible mechanisms may exist for generating pGE. Senescent fibroblasts are known to undergo methylation changes (Cruickshanks et al. 2013), and these alterations may lead to epigenetic alterations that promote stochastic changes in gene expression. Alternatively, it has been suggested that DNA damage may modulate gene expression by altering the binding capacity of transcription factors (Rose et al. 2012).

Interestingly, the reprogramming of fibroblasts into induced pluripotent stem cells (iPSCs) via the addition of OCT4, SOX2, KLF4 and MYC (OSKM) requires a long stochastic phase of gene activation associated with changes in histone modifications at somatic genes and activation of DNA repair and RNA processing (Buganim et al. 2013). This stochastic gene expression may be the result of “promiscuous binding” by OCT4, SOX2 and KLF4, where they occupy accessible chromatin and bind to promoters of genes that are active or repressed (Buganim et al. 2013). It is possible that pGE

in senescent cells partly mimics stochastic gene activation associated with cellular reprogramming. However, whether pGE in senescent cells is associated with factors that can undergo “promiscuous binding” has yet to be determined.

Whether pGE plays a functional role in cell senescence has yet to be determined. However, it can be speculated that pGE may function to generate an array of tissue-restricted proteins that can subsequently be processed into peptides by autophagic proteases for presentation on MHC molecules (Dengjel et al. 2005). Similar to the presentation of tumour-associated antigens (Reuschenbach et al. 2009), senescent cells may also present antigens that can be recognized by immune cells, thereby becoming antigen-presenting cells (APCs). Although the up-regulation of MHC molecules on senescent cells have yet to be fully evaluated, the up-regulation of MHC class I but not MHC class II in response to DNA damage in fibroblasts has been reported (Tang et al. 2014). It remains to be determined whether pGE is a component of immunogenic conversion.

Atypical senescent states

TGF β -induced senescence

A growing body of evidence suggests that the members of the transforming growth factor beta (TGF- β) family can induce a senescence-like state. Experimentally, senescence has been predominantly, but not exclusively, characterized by the presence of senescence-associated beta galactosidase (SA- β -Gal) staining and the up-regulation of cyclin-dependent kinase inhibitors (CDKi) (see below). Human prostate basal cells treated with TGF- β 1/2/3 show increased SA- β -Gal activity, which is associated with the flattened, and enlarged cell morphology typical of adherent senescent cells in vitro (Untergasser et al. 2003). Similarly, TGF- β 1 has been reported to induce a senescent state in bone marrow mesenchymal stem cells as a result of increased mitochondria ROS production (Wu et al. 2014). These cells also showed SA- β -Gal staining and an increased expression of p16. Yu et al. (2010) demonstrated that TGF- β 2 could induce a senescent-like state in human trabecular meshwork cells. Again, this was associated with SA- β -Gal staining, increased levels of p16 at both the message and protein level and a reduction in the

level of pRB protein. No impact on p21 mRNA or protein expression was observed in response to TGF- β 2 exposure. Other groups have also reported a role for TGF- β signaling in inducing a senescent state (Senturk et al. 2010; Minagawa et al. 2010; Acosta et al. 2013).

It is generally accepted that SA- β -Gal staining should be used in conjunction with several other senescent markers, as it does not appear to detect senescent cells specifically (Severino et al. 2000). However, other than the expression of CDKi, it appears that the phenotypes of cells induced to enter senescence by exposure to TGF- β s have been poorly characterized, especially in regard to immunogenic conversion. Some cell types that become senescent via this route may be cleared by the immune system in a manner analogous to those undergoing developmentally programmed senescence (qv). Others may not, and this area represents a fruitful field for further investigation.

Developmentally programmed senescence

Cells sharing features of senescence have been reported within the mesonephros and the endolymphatic sac of the inner ear in human and mouse embryos, as well as the neural roof plate and apical ectodermal ridge in rodents (Munoz-Espin et al. 2013; Storer et al. 2013). The authors hypothesize that this “developmental senescence” (DS) is a programmed part of normal embryonic development. DS was demonstrated experimentally by the presence of SA- β -Gal activity and senescence-associated heterochromatin (Munoz-Espin et al. 2013). These cells seem to lack detectable DNA damage and appear to have become senescent independent of p53 and p16 and have gene expression patterns that significantly overlap with those of IMR90 fibroblasts in a state of oncogene-induced senescence. Arrest in this instance is dependent instead upon p21, regulated via the TGF- β /SMAD and PI3K/FOXO pathways (thus showing some affinity with other TGF- β induced senescent states). Interestingly, DS cells are removed during normal embryonic development by macrophages in a manner related to immune clearance of senescent cells in the mature organism (or by apoptosis should senescence fail) contributing to the formation of normal tissue architecture. Thus, the long-recognized distinction between programmed cell death in development and apoptosis in the mature organism appears to be mirrored in DS. Given that the expression of p21 in developing

embryos is often attributed to ‘terminal differentiation’ (Vasey et al. 2011), it will be interesting to determine how many of these p21 positive cells are senescent cells and have undergone immunogenic conversion.

Metabolic stress-induced senescence

Metabolic stress, defined here as a combination of aerobic glycolysis and mitochondria dysfunction can potentially trigger a senescent state. All organisms that use aerobic glycolysis form reactive acyclic α -oxoaldehydes (e.g. methylglyoxal and glyoxal) spontaneously from triosephosphates and by a wide variety of other routes (Thornalley 2008). These dicarbonyl compounds are highly reactive and damage proteins through non-enzymatic modification producing a wide variety of covalent adducts (AGEs). Elevated levels of methylglyoxal and glyoxal are known to be cytotoxic and although the mechanism of action remains imprecisely defined, it can be blocked by ROS scavengers, suggesting that oxidative stress mediates at least some of the deleterious effects (Shangari and O’Brian 2004).

Cytosolic and mitochondrial protection from dicarbonyl damage is primarily mediated through the action of the glyoxalase system that consists of two enzymes, glyoxalase I and II. However, in cultures of WI38 fibroblasts, a significant reduction in the activity of glyoxalase-I occurs with serial passage (Ahmed et al. 2010). Treatment of cultures of ASF2 human adult dermal fibroblasts with micro or millimolar concentrations of glyoxal or methylglyoxal renders them senescent within 72 h. This was defined by the presence of typical senescent morphology, irreversible growth arrest and increased SA- β -Gal activity (Sejersen and Rattan 2009). Further studies (Larsen et al. 2012) extended these observations to immortalized human mesenchymal stem cells (MSCs) and demonstrated that treatment with physiologically reflective (Han et al. 2007) concentrations of glyoxal for 72 h led to senescence without significant cell death (although massive cell death occurred at higher glyoxal concentrations). Elevated levels of SA- β -Gal, p16 and DNA damage (as measured by COMET) accompanied the growth arrest. Interestingly, a profound reduction in the ability of these senescent MSCs to differentiate into functional osteoblasts (as determined by alkaline phosphatase and mineralization assays) was also observed. Given the imbalances in glucose metabolism that accompany mammalian ageing (and diabetes), the authors proposed that this type of

metabolic stress might underlie age-related changes in bone function. Unfortunately, no markers of immunogenic conversion have yet been measured in this system and whilst the presence of DNA damage could indicate the likelihood of a secretory response, this cannot be assumed. Thus, the propensity of senescence human MSCs to be cleared by the immune system remains unknown and is of considerable physiological significance.

In CD8⁺ T cells, senescence can occur by a number of routes including AMPK driven activation of p38-MAP kinase leading to the inhibition of telomerase activity and proliferation (Lanna et al. 2014) and exposure to prostaglandin E2. Such senescent CD8⁺ T cells arrest with defective mitochondria. Inhibition of p38 MAPK signaling partially restores this and increases proliferation (Henson et al. 2014). An important question, given that senescence probably evolved to facilitate immune clearance, is how the immune system deals with its own senescent cells. As a special case, we have excluded immune cells from our discussion of immunogenic conversion, but note the need for subject specialists to address this question.

Endoplasmic reticulum stress-induced senescence

Endoplasmic reticulum (ER) stress may also promote a senescent-like response. The accumulation of unfolded proteins in the ER triggers a stress-signaling pathway that can result in cell cycle arrest mediated by p27 (Han et al. 2013) and the p53/47 isoform (Bourougaa et al. 2010). Furthermore, ER stress has also been shown to induce an inflammatory response via NFκB activation (Garg et al. 2012) and induce cytokines such as MCP-1, IL-6 and IL-8 (Schroder 2008), which are capable of attracting and activating immune cells (Sagiv and Krizhanovsky 2013). ER stress has also been shown to promote cell survival, another feature of cell senescence (Raciti et al. 2012). Interestingly, a senescent state via activation of ER stress-dependent p21 signaling has been reported in proximal tubular epithelial cells, triggered by receptors for advanced glycation end-products (RAGE) (Liu et al. 2014). Although, ER stress-induced senescence has the potential to induce an immunogenic phenotype in the absence of DNA damage, a full evaluation of the phenotype is required to determine if this is so.

Experimental induction of cyclin-dependent kinase inhibitors

For many researchers, irreversible cell cycle arrest is the canonical trait of senescent cells. Such growth arrest can be induced experimentally by the up-regulation or over-expression of cyclin-dependent kinase inhibitors (CDKi). Thus, valuable models are, at least potentially, available in which to study the physiological effect of growth arrest distinct from the DDR or any other upstream response. Unfortunately, there has been little characterization of the phenotype of cells rendered 'senescent' by this means.

Blagosklonny and co-workers (Korotchikina et al. 2009) used an isopropyl-thio-galactosidase (IPTG)-inducible p21 expression construct to induce a senescence-like state in an HT1080-derived cell line (HT-p21-9). Characterization of the phenotype of these cells does not appear to have been attempted beyond observing irreversible growth arrest and the presence of increased SA-β-Gal activity. Given that HT1080 is a highly tumorigenic fibrosarcoma carrying an activated N-ras oncogene (Benedict et al. 1984), it probably represents a poor genetic background in which to assess whether markers of immunogenic conversion or resistance to cell death can be induced by CDKi overexpression alone. However, the basic principle of using such a construct for that purpose is sound.

Tokarsky-Amiel et al. (2013) showed that overexpression of p14^{ARF} in the epidermis of the skin of mice (using a tetracyclin-inducible construct) resulted in mass apoptosis and cell cycle arrest. As measured by SA-β-Gal activity, the p14^{ARF} transgene drove senescence in up to 8 % of the surviving cells in the epithelium by a p53-dependent mechanism (demonstrated by ablation of p53 through co-expression of a specific shRNA directed against it). These senescent cells were viable within the epidermis for several weeks consistent with lack of clearance. Unfortunately, minimal analysis of their phenotype was conducted (beyond assessment of the message levels for the senescence-associated genes *Pai-1* and *Dcr2*). Thus, the immune state of the p14^{ARF}-senescent cells is currently unclear, and the picture is complicated by the fact that senescent rodent cells do not display a senescent secretome under some conditions. However, given that alopecia and follicular stem cell dysfunction were observed in the animals, it is clear that cells rendered 'senescent' in this manner can exert phenotypic effects. Thus, there is some evidence that cell cycle arrest alone may be sufficient to cause problems in highly mitotic tissues such as the epidermis, but large amounts of work remain to be done.

Table 1 Phenotypic differences between sterile senescence and immunogenic senescence

Phenotype	Sterile senescence	Immunogenic senescence
DNA damage	Unlikely	Likely
CDKi expression (i.e. p16)	Yes	Yes
Inflammatory response	No	Yes
Immune ligand expression	No	Yes
Promiscuous gene expression	Unknown	Likely
Pro-survival response	Unknown	Likely

CDKi overexpression systems clearly have the potential to be valuable tools. However, the extent to which these are physiologically reflective can legitimately be challenged. This can be understood in two ways (i) the mechanism by which the growth arrest is induced has not been reported *in vivo* and (ii) cells do not become senescent *en mass* but gradually as a result of tissue turnover throughout life. Thus, findings made with these systems could be considered ‘artefactual’.

By way of addressing these concerns, it is worth remembering that for many years, replicative senescence was dismissed as a ‘tissue culture artefact’ because senescent cells had not been observed *in vivo* (evidence for their existence in tissue remained severely limited until the late 1990s). By the same token, elevation of CDKi alone in cells *in vivo* is not impossible. Absence of evidence is never evidence of absence. Similarly, many overexpression systems model systems can be said to be non-physiological. However, valuable data is routinely gathered using them and in this instance could allow researchers to gauge the maximum physiological impact that irreversible growth arrest can have on tissue function. Thus, if these limits are recognized, such models are potentially utile, especially when combined with detailed analysis of phenotypes known to exist in other ‘senescent cells’ (e.g. apoptosis resistance, immune ligand presentation and the secretory response).

Concluding remarks

Historically, the primary interest of researchers studying cell senescence was irreversible cell cycle arrest. However, it is now apparent that senescent cells can also display phenotypes that function to promote self-elimination by the immune system. Whilst many questions remain

unanswered around the mechanistic basis of immunogenic conversion, the DDR probably plays a central role. However, some senescent states appear to avoid immunogenic conversion for reasons that are currently unclear. In addition, immunogenic conversion caused by other mechanisms (such as ER stress) cannot be ruled out.

Experimental demonstration that cells from a particular tissue and/or species have entered irreversible cell cycle arrest distinct from terminal differentiation was once enough to label them ‘senescent’. This led to the unfortunate tendency to extrapolate aspects of their phenotype, sometimes unstudied and sometimes wholesale, from the data on senescent human fibroblasts (if not in the primary reports then in secondary sources attempting a critical synthesis).

Compared to the secretory response, immune ligand expression, apoptosis resistance (and possibly pGE), cell cycle arrest may prove to be a minor physiological phenotype considered in terms of the impact that senescent cells have in living tissues. Thus, when studying novel cell types, cells from new animal species and using new triggers of senescence, the observation of cell cycle arrest may be a good start, phenotypically speaking, but a bad end. Much more detailed characterization is probably necessary, focusing on the aspects of the senescent phenotype we discuss above.

Given the various states of ‘cell senescence’, across many different cell types in multiple species, some division of the semantic domain covered by “cell senescence” is probably helpful. Accordingly, we propose two working subcategories for senescence in non-immune cells, (1) immunogenic senescence, referring to irreversible cell cycle arrest accompanied by a phenotype promoting self-elimination by the immune system and (2) sterile senescence, referring to irreversible cell cycle arrest that does not evoke an immune response (see Table 1). In this model, a key distinction between senescent cells is thus not ‘how has it stopped?’ but rather ‘what has it started?’

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