Review Article

The Importance of Senescence in Ionizing Radiation-Induced Tumour Suppression

(review / senescence / ionizing radiation / cytokine expression)

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Abstract. Cellular senescence is a condition of long-lasting proliferation arrest, induced in cells in response to various stressors. These stressors include telomere shortening and/or dysfunction, DNA damage, and oncogene signalling. Epithelial and mesenchymal cells and also tumour cells derived from these tissues are more resistant to radiation-induced apoptosis and respond to irradiation mainly by senescence. Senescence-associated molecular mechanisms related to the activation of canonical DNA damage pathway ATM-p53 as well as mechanisms related to the extracellular signals, cytokine increase and upregulation of their receptors are discussed in this review.

Introduction

The damage to cells by ionizing radiation mainly includes modifications of DNA, the most devastating of these being double-strand breaks (DSB). It is known that in response to DNA damage by ionizing radiation, three protein kinases from the family of phosphoinositide-3-kinases are quickly activated: ataxia-teleangiectasia mutated (ATM), ataxia-teleangiectasia and Rad3-related (ATR), and catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Bakkenist and Kastan, 2003). ATM and ATR are both extremely large kinases (350 and 301 kDa, respectively), which upon activation phosphorylate many substrates and through them trigger repair or apoptosis, necrosis, mitotic catastrophe, and stress-induced premature senescence (SIPS).

Very early changes were also detected in chromatin flanking the DSB. In the site of a nascent DSB the histone H2AX is quickly (within minutes) phosphorylated by ATM kinase. This phosphorylation spreads to adjacent chromatin. To the modified chromatin are consequently bound other proteins, such as Mdc1 or 53BP1, and discernible foci called ionizing radiation-induced

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Abbreviations: 53BP1 – tumour protein p53-binding protein 1, ATM – ataxia-telangiectasia mutated kinase, ATP – adenosine triphosphate, ATR – ATM and Rad3-related kinase, BRCA1 – breast cancer 1, Cdc6 – DNA replication licensing factor, CDK – cyclin-dependent kinase, Chk2 – checkpoint kinase 2, CXCL1, 2, 3, 5, 6, 7 – chemokine (C-X-C motif) ligand 1, 2, 3, 5, 6 and 7, also known as GRO α, β, γ, ENA-78, GCP2, NAP2, respectively), CXCR1 and CXCR2/IL8RB – GPCR family chemokine receptors , DNA-PKcs – DNA-dependent protein kinase catalytic subunit, DSB – double-strand break, E2F – transcription factor E2F, γH2AX – γ variant of histone H2AX, H2AX – histone H2AX, HDAC4 – histone deacetylase 4, IL-1, -6, -8 – interleukin 1, 6, 8, IRIF – ionizing radiation-induced foci, JAK1 – member of Janus family tyrosine kinases, MAPK – mitogen-activated protein kinase, Mdc1 – mediator of DNA damage checkpoint 1, MCP-1 – chemotactic protein 1, MMP – matrix metalloproteinase, MMP1, 3, 10 – matrix metalloproteinase 1, 3 and 10, Mos – activator of the mitogen-activated protein kinase pathway, Mre11 – meiotic recombination 11 homologue A, MRN – Mre11-Rad50-Nbs1 complex, Nbs1 – Nijmegen breakage syndrome protein 1, NFκB – nuclear factor κB, OIS – oncogene-induced senescence, $p15^{INK4b}$ – cyclin-dependent kinase inhibitor 2B, $p16^{INK4a}$ – cyclin-dependent kinase inhibitor 2A, $p21^{WAF1/Cip1}$ – cyclin-dependent kinase inhibitor 1A, p53 – tumour suppressor protein 53, PCNA – proliferating-cell nuclear antigen, PI3K – phosphatidylinositol 3-kinases, Rac – subfamily of the Rho family of GTPases, Rad 50 – Rad 50 homologue, Ras – protein subfamily of small GTPases, Rb – retinoblastoma protein, RS – replicative senescence, SA-β-gal – senescence-associated β-galactosidase, SIPS – stress-induced senescence, STAT1 – signalling protein of JAK1 kinase, TGFβ – transforming growth factor β, Tip60 – K (lysine) acetyltransferase 5, also known as Kat-5.

foci (IRIF) are formed. In these foci many proteins related to repair or death processes are recruited, among them ATM kinase, Mre11, Rad 50, Nbs1 (proteins of DNA repair complex MRN), Mdc1, 53BP1, Tip60, HDAC4 and BRCA1. All these proteins can surround DSB in all phases of the cell cycle, including interphase. Interactions of these proteins play a crucial role in DSB processing and repair (Bekker-Jensen et al., 2006).

In our previous studies we showed that lymphocytes isolated from peripheral blood of healthy donors and irradiated *in vitro* by the doses up to 10 Gy die after irradiation by programmed cell death – apoptosis (Vilasová et al., 2008). Also cells of leukaemic cell lines (HL-60, MOLT-4) die after irradiation with a single dose by apoptosis (Vávrová et al., 2001, Řezáčová et al., 2008). It is clear that healthy lymphocytes as well as tumour cells of haematopoietic origin are removed after irradiation by apoptosis, programmed cell death, which – contrary to necrosis – is an active process and requires energy in the form of ATP. On the other hand, cells of mesenchymal origin show a considerable resistance to the apoptosis induction. Our own research proved that stem cells isolated from dental pulp are resistant to radiation-induced apoptosis (Muthná et al., 2010). Similar behaviour was observed after the irradiation of fibroblasts (Suzuki et al., 2001; Suzuki and Boothman, 2008). Instead of apoptosis induction, these cells enter a state of permanent cell cycle arrest, known as senescence. Cellular senescence is characterized by a cell cycle arrest, which effectively inhibits proliferation. Senescent cells do not divide, cannot form colonies and exhibit a broad spectrum of morphological changes.

Three distinct types of senescence are recognized:

1/ replicative senescence
2/ stress-induced senescence, also known as premature senescence or stress-induced premature senescence
3/ oncogene-induced senescence.

Replicative Senescence (RS)

During the first half of the 20th century it was generally believed that isolated cells are immortal in cell culture and can divide infinitely. These beliefs were mainly based on experiments and ideas of Alexis Carrel, who established an immortal line of fibroblasts isolated from the heart of a chick embryo, which kept proliferating for over decades (Carrel, 1928). However, although immortalized mutated cell lines were obtained by others, Carrel's experiments with differentiated cells could not be reproduced. Finally, in 1961 Leonard Hayflick and Paul Moorhead reported that human and rodent cells derived from embryonic tissues can only divide a finite number of times in culture (Hayflick and Moorhead, 1961). Their experiment shows that the cells then no longer proliferate, but remain living in a state described as cellular senescence. The phenomenon is known today as Hayflick's limit.

This type of senescence is so-called replicative senescence (RS). Approximately after fifty population doublings of the somatic cells the proliferation stops, the number of cells in S phase of the cell cycle decreases and the cells are irreversibly arrested in G1 or rarely in G2 phase of the cell cycle. It was observed (Harley et al., 1990) that the mean telomere length decreases during the serial passage of normal human diploid fibroblasts. During each division in average 50 base pairs are lost from the end of the chromosome (Levy et al., 1992). Since in normal postnatal somatic cells this loss is not replenished, RS is caused by progressive telomere shortening during each DNA replication.

Senescent cells remain metabolically active (Ouellette et al., 2000), and are characterized by expression of senescence-associated β-galactosidase (SA-β-gal) activity at pH 6 (Dimri et al., 1995). Dysfunctional telomeres trigger the DNA-damage response pathway, including activation of ATM, 53BP1, Mdc1, Chk2, and H2AX (d'Adda di Fagagna et al., 2003; Herbig et al., 2004). Also gene expression is altered, including up-regulation of cell cycle inhibitors $p21^{Cip1/Waf1}$ and $p16^{INK4a}$, down-regulation of cell cycle proteins PCNA, cyclins A and B. The up-regulation of p16 seems to be independent of the ATM-p53 pathway (Herbig et al., 2004). Another typical hallmark of senescent cell is inhibition of E2F due to hypophosphorylation of Rb (Pazolli and Stewart, 2008).

Stress-Induced Senescence (SIPS)

In many types of cells (fibroblasts, endothelial cells, melanocytes) the prevailing response to various DNA-damaging stressors, such as ionizing and UV radiation, hydrogen peroxide, or chemotherapeutic is not cell death, but so-called stress-induced senescence. Contrary to the replicative senescence, stress-induced senescence (SIPS) is not related to telomere shortening. The molecular mechanisms of SIPS are almost identical to those of RS. The major player is classical DNA-damage response mediated by ATM-p53-p21. The cells which enter SIPS often contain DNA damage-associated foci with γH2AX and other typical proteins (see above). It is presumed that these foci indicate irreparable DSBs. This in turn leads to constitutive signalling to activate p53. Activation of p53 results in an increase in $p21^{Cip1/Waf1}$ levels and cell-cycle arrest. The up-regulation of $p16^{INK4a}$ shows a delayed onset in comparison to ATM-p53-p21.

What do we know about SIPS induced by ionizing radiation? One of the best characterized responses to radiation-induced DNA damage is IRIF formation, activation of ATM kinases, and consecutive increase and phosphorylations of p53. IRIF formation peaks within 30 min after irradiation. The activation of ATM-p53 pathway is rapid and occurs in minutes after irradiation. Subsequent kinetics depends on the cell type and received dose, and seems to be related to the fate of the cell.

In the cells of haematopoietic origin the activating phosphorylations of p53 disappear within hours after irradiation after exposure to a single lethal dose. For ex-

ample, in the cells of human T-leukaemia cell line MOLT-4 the increase in p53 and the phosphorylations of p53 on serine 15 and serine 392 are observed 0.5–6 h after the irradiation by the dose of 7.5 Gy (absolutely lethal dose); 8 h after the irradiation the phosphorylation on serine 15 decreases and phosphorylation on serine 392 almost disappears. This is followed by apoptotic cell death 24–72 h after irradiation (Szkanderová et al., 2003; Tichý et al., 2007). After exposure to lower doses of radiation (1–3 Gy), part of the MOLT-4 population also die by apoptosis, while the rest of the cells repair the damage and re-enter cell cycle. Only less than 10 % of surviving MOLT-4 cells retain IRIF 72 h after irradiation by the dose of 1.5 Gy (Řezáčová et al., 2008).

On the other hand, cells of epithelial origin, such as human lung carcinoma cells (Suzuki et al., 2001) or human breast carcinoma cells (Jones et al., 2005), or cells of mesenchymal origin, such as embryonic fibroblast-like cells (Suzuki et al., 2006) or dental pulp stem cells (Muthna et al., 2010), react to irradiation preferentially by induction of SIPS and thus permanent cell cycle arrest. The induction and transactivation of p53 lasts at least 10–13 days after irradiation. Also up-regulation of $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ is continuously observed days after irradiation in these cells (Suzuki et al., 2001, 2008; Muthna et al., 2010). IRIF are formed swiftly upon irradiation, and most of them disappear within 24 h. However, some IRIF are observed after this period in nearly all cells irradiated with high doses (Suzuki et al., 2006). It is believed that these foci are an indicator of irreparable DSB. The persisting IRIF constitute a site with continuous activation of DNA-damage response, leading to the observed induction of p53-p21 pathway.

While the exact mechanism which decides between apoptosis/cell death and SIPS is not yet well understood, we know that the crucial molecule of permanent cell-cycle arrest during SIPS (and also RS) is Rb protein. The role of Rb protein is to negatively regulate transcription factor E2F. E2F is necessary for activation of transcription of S phase-specific genes. Unphosphorylated Rb protein forms a complex with E2F and thus prevents interaction of E2F and DNA. After phosphorylation of Rb protein by cyclin-dependent kinase, the transcription factor E2F is released from Rb and activates gene transcription. If cyclin-dependent kinases are inhibited, Rb is hypophosphorylated, the release of E2F is insufficient for cell-cycle progression and senescence is initiated. Known inhibitors of cyclin-dependent kinases involved in SIPS initiation include $p21^{Cip1/Waf1}$, $p16^{INK4a}$, and $p15^{INK4b}$. The up-regulation of CDK2 and CDK4 inhibitor $p21^{Cip1/Waf1}$ is related mainly to ATM-p53 DNA-damage response. Protein $p16^{INK4a}$, inhibitor of cyclin-dependent kinases CDK4 and CDK6, can also cause Rb hypophosphorylation and cell senescence (Dimri, 2005), but it is not a universal hallmark of all senescent cells. The mechanisms of $p16^{INK4a}$ up-regulation remain to be elucidated. Another cyclin-dependent kinase inhibitor – $p15^{INK4b}$ – was identified as marker of Ras-induced senescence (Collado et al., 2005).

Oncogene-Induced Senescence (OIS)

The longevity of a multicellular organism depends on its ability to renew and regenerate tissues. However, this ability on the other hand can trigger uncontrollable proliferation and cause oncogenic lesions (Kuilman et al., 2008). OIS can be triggered by aberrant mitogenic and/or oncogenic signals through Ras, Mos, Cdc6, cyclin E, STAT5, etc. (Novakova et al., 2010). It is presumed that the main role of oncogene-induced senescence (OIS) is to elicit an anti-tumour barrier, which can exclude early neoplastic cells from the proliferating pool. Indeed, markers characteristic for senescent cells were found in pre-invasive stage of human tumours (Bartkova et al., 2006; Acosta, 2008; Acosta et al., 2008; Kuilman et al., 2008). While the main role in RS and SIPS is attributed to proteins p53 and Rb, and these pathways are also involved in the induction of OIS (Bártková et al., 2005; Gorgoulis et al., 2005), other studies revealed that the crucial mechanism of OIS induction is related to an increase in secretion of pro-inflammatory cytokines and/or their receptors (Acosta, 2008; Kuilman et al., 2008). The importance of pro-inflammatory cytokines IL-6 and IL-8 (Kuilman et al., 2008) for the ability of tumour cells to enter OIS and the increase in IL-1α, IL-6, IL-8, GRO α, β, and γ, GCP2, NAP2, ENA-78 (Acosta, 2008) during OIS induction were discovered. These interleukins belong to the family of chemokines – small chemotactic cytokines, which are responsible for communication among various cells and have multiple functions in both healthy and diseased organism. Chemokines interact with the target cells via receptors of the G protein-coupled receptor superfamily. The increase in these receptors is also linked with senescence. Acosta et al. (2008) show that an increase in CXCR2/IL8RB contributes to OIS induction. Thus, the expression of CXCR2 is increased in OIS cells, as well as the expression of all of its known ligands – IL-8, GRO α, β, and γ, GCP2, NAP2, ENA-78.

In response to oncogenic stress the genes for IL-6 and IL-8 are activated by transcription factor C/EBPβ. IL-6 secretion is associated with activation of the ATM-Chk2 pathway, independent of p53 (Coppé et al., 2010). The increase in IL-8 correlates with the increase in p16 in colorectal carcinoma cells undergoing cell-cycle arrest. Another inhibitor of cyclin-dependent kinase $p15^{INK4b}$ is increased under the influence of IL-6 (Acosta, 2008). Senescence induced through activation of CXCR2 is at least partially dependent on p53. Activation of CXCR2 results in activation of NFκB, MAPK, PI3K and Rac. Rac in turn increases production of reactive oxygen species by NADPH oxidases, which provoke or sensitize to DNA damage (Acosta et al., 2008).

The senescent cells are thus characterized by increased expression of receptor CXCR2 and by increased secretion of various cytokines, proteases and matrix components, including IL-1, IL-6, IL-8 and other CXCR2 ligands. Activation of the receptor through intracellular signalling induces production of ROS and triggers

DNA-damage response mediated by p53. Increased CXCR2 was also found on pre-neoplastic cells (Acosta et al., 2008). The findings support the idea of senescence, and the DNA-damage pathway activation acts as an anti-tumorigenic barrier during early stage of neoplastic lesions (Bartek et al., 2008). On the other hand, increased secretion of pro-inflammatory cytokines, proteases and matrix components can also promote proliferation and invasiveness of malignant cells and promote tumour development (Coppé et al., 2010).

Recently, induction of a similar secretory phenotype as accompanies OIS was reported in cells undergoing stress-induced senescence (Coppé et al., 2010; Novakova et al., 2010). The spectrum of induced cytokines was slightly different, including involvement of interferon β-STAT1 axis. However, neither IL-6 nor the JAK1/STAT1 signalling was strictly required for drug-induced senescent phenotype. This suggests that not only activation of DNA-damage signalling and hypophosphorylation of Rb, but also changes in the cytokine secretory profile are shared by all major types of senescence.

Detection of Senescent Cells

Dimri et al. (1995) observed increased activity of β-galactosidase at pH 6 in senescent cells, a marker now known as "senescence-associated β-galactosidase activity" (SA-β-gal). The increase in SA-β-gal is attributed to increase in lysosomal activities, including great increase in lysosomal β-galactosidase (Lee et al, 2006). This enzyme, present in all cells, has pH optimum around 4, but when the activity is greatly increased during senescence, it becomes detectable also at pH 6. *In situ* SA-β-gal activity has been widely used as a biomarker of senescence, the method using 5-bromo-4-chloro-3-indoyl-β--D-galactopyranoside (X-gal) as a substrate that is cleaved to blue-coloured product. Debacq-Chainiaux et al. (2009) developed a new method based on the abundance of the lysosomal enzyme. The method uses alkalinization of lysosomes followed by the use of 5-dodecanoylaminofluorescein di-β-D-galactopyranoside – a fluorogenic substrate for β-gal. This fluorescence-based method is more sensitive and better for quantification, and can be adopted for flow cytometry. Senescent cells also exhibit many other typical characteristics: cell-cycle arrest, morphologic changes, increased expression of cell-cycle inhibitors (e.g. $p21^{Cip1/Waf1}$, $p16^{INK4a}$, $p15^{INK4b}$), hypophosphorylation of Rb, activation of DNA-damage response, and senescence-associated secretory phenotype.

Conclusions

Senescence can be triggered by various internal and external insults or signals. These factors include telomeric dysfunction due to repeated divisions (replicative senescence), severe irreparable DNA damage and chromatin disruption (stress-induced premature senescence) and expression of oncogenes, such as Ras (oncogene-induced senescence). Regardless of the triggering signal, senescent cells share many typical characteristics – cell-cycle arrest, increased expression of cell-cycle inhibitors ($p21^{Cip1/Waf1}$, $p16^{INK4a}$, $p15^{INK4b}$), hypophosphorylation of Rb, activation of DNA-damage response, senescence-associated secretory phenotype, morphologic changes, and increase in SA-β-gal activity. The main feature of senescence is the inability of cells to enter cell cycle, even when stimulated by growth factors. Senescent cells remain metabolically active and their gene expression and secretory profile are changed. Epigenetic changes, such as characteristic heterochromatin foci rich in histone H3 trimethylated on lysine 9, are also observed (Adams, 2007).

It is generally accepted that cellular senescence is a tumour-suppressive mechanism. In early stage of preoncogenic lesions, internal mechanisms (including increase in p53 and p21) are activated in the cells to induce senescence and remove the dangerous cells from a proliferating pool. In parallel, senescence can be triggered by extracellular signalling molecules produced, including Wnt family members, transforming growth factor β (TGF-β), plasmin and interleukins. TGF-β was shown to induce senescence in keratinocytes through v-Ras and induction of cyclin-dependent kinase inhibitors $p16^{INK4a}$ and $p15^{INK4b}$ (Kuilman et al., 2009). Other cytokines (e.g. IL-1, IL-6, IL-8, GRO α) can also trigger senescence, and a very important role in this induction is assigned to CXCR2 receptor (Acosta, 2008).

On the other hand, senescent cells themselves secrete various factors, regardless of the initial stimulus that triggered senescence. The phenomenon is known as senescence-associated secretory phenotype. The factors secreted by cells in RS, SIPS and OIS include pro-inflammatory cytokines (IL-1, IL-6), chemokines (IL-8, GRO, MCP-1, etc.), growth factors (EGF, VEGF, colony-stimulating factors, etc.), matrix metalloproteinases (MMP1, 3, 10) and their inhibitors, plasminogen activators and their inhibitors, and fibronectin. It was observed that these factors secreted by senescent cells can promote tumour growth and invasiveness, mainly of epithelium-derived tumour cells, as well as angiogenesis. The role of altered secretion profile of senescent cells thus seems to be controversial so far. We can speculate that in the short term and early tumour stage the secreted factors provide alarm signals and recruit immune cells, which allows removal of damaged cells and tissue repair, but if the response fails, then in the long term these factors can by contrast promote tumour growth and invasiveness.

How does this knowledge apply to radiotherapy of tumours? We now know that the majority of solid tumour cells respond to radiotherapy by senescence, and that induction of apoptosis is a much rarer event. Radiation-induced senescence of tumour cells effectively blocks the proliferation of malignant cells and therefore inhibits tumour growth. On the other hand, senescent cells derived either from the tumour itself or from normal stroma cells remain metabolically active and se-

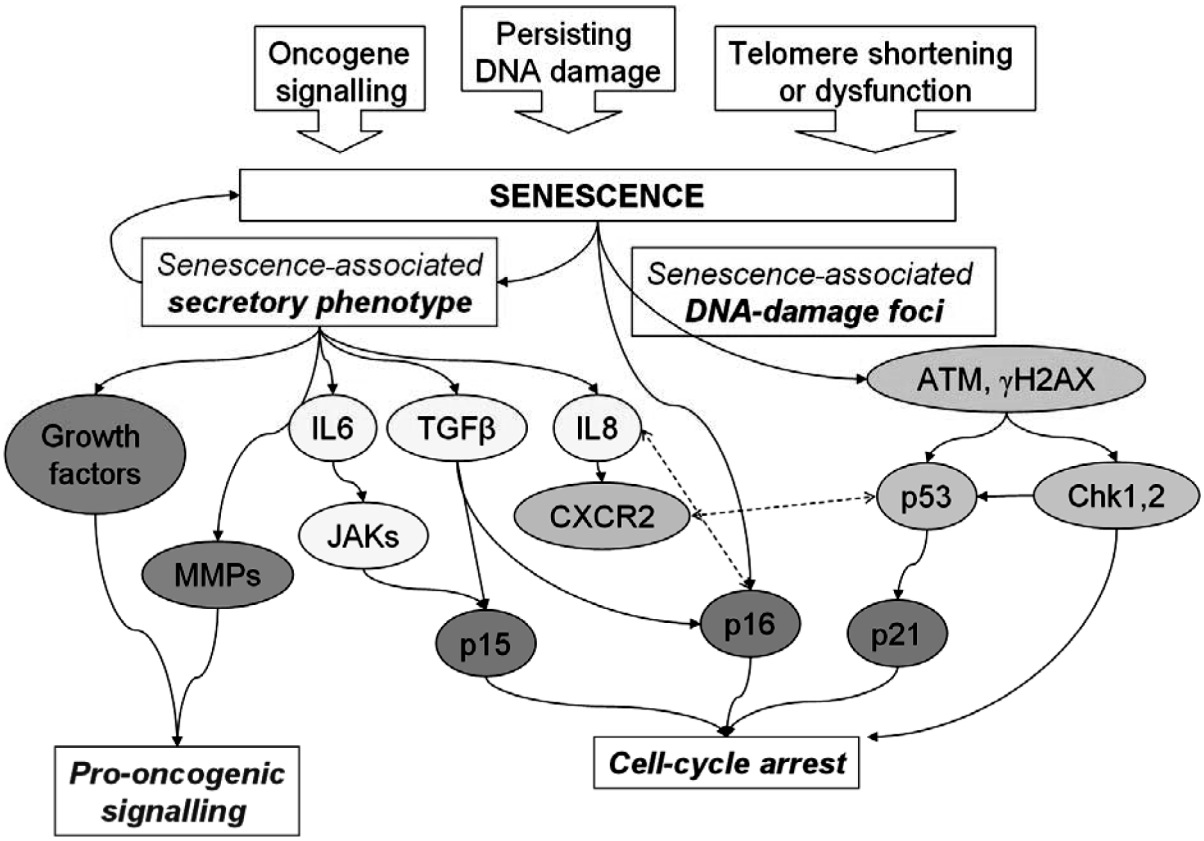

Fig. 1. Mechanisms of senescence. Regardless of the initial trigger, senescent cells share similar characteristics. The classic pathway is initiated by DNA-damage sensors resulting in phosphorylation of kinase ATM, increase in p53 and subsequent increase in cyclin-dependent kinase inhibitor – p21. The increase in p53 is also involved in cytokine-induced senescence initiated by IL-8 and GRO1 through activation of their receptor CXCR2. Also other cytokines secreted by senescent cells promote further senescence induction: TGF-β activates adaptor proteins of the SMAD family and contributes to the increase in cyclin-dependent kinase inhibitors p15 and p16; IL-6 activates MAP kinases JAK that contribute to the increase in p15. The secretory profile of senescent cells is altered; these cells produce various pro-inflammatory cytokines, growth factors and MMPs, which in later stage can promote tumorigenesis.

crete various factors. These factors can in the long term support radioresistance, tumour growth and invasiveness. It is therefore essential to better elucidate senescence mechanisms and general DNA-damage response signalling in epithelial cells and their crosstalk. Future deeper understanding of these mechanisms could ensure permanent destruction of tumour cells, while protecting healthy tissues during radiotherapy.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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