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Telomeres, senescence and cellular radiation response

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Abstract. Telomeres shield the ends of chromosomes from degradation and end-to-end fusions. They shorten at each cell division and when they reach a critically short length, cells arrest in the G1 phase of the cell cycle and undergo senescence. This effectively limits the proliferative potential of cells. Senescence functions as a tumour suppressor mechanism and appears to contribute to the process of ageing. If senescence is circumvented by tumour viruses, proliferation is re-initiated until cells enter crisis. Activation of telomerase prevents telomere attrition and cells become immortal. Cellular response to ionizing radiation involves induction of cell cycle checkpoint arrests and programmed cell death. Because radiation produces double strand breaks in DNA, which cause telomere-less chromosome ends, radiation response appears to be the result of inappropriate induction of cellular senescence mechanisms.

Key words. Telomeres; telomerase; senescence; ageing; radiation; programmed response.

Cellular response and radiation action

The response of cells to the presence of genome damage is programmed [1]. It follows a number of principles common to all cellular phenomena. An understanding of these principles provides insight into how cells function. Protein interactions are the basis of programmed cellular response. Wherever biological processes have been studied at the molecular level, protein signalling networks are evident. These follow the principles of cybernetic control systems, characterized by four features, shown in table 1 [2, 3]. Signals are a special form of information. As opposed to static, structural information, signals circulate in cybernetic systems [3]. Such systems of intricate cellular biochemistry form molecular automata. They comprise a collection, or 'body', of proteins and other accessory cellular components which control and execute biological responses and could be referred to as cybersomes. The repertoire of signals to which a cell responds does not normally include genome damage caused by ionizing radiation. Nevertheless, response to radiation exposure is programmed and is based on activation and transduction of biochemical signals within cybernetic protein systems. There are two consequences: a) programmed response to radiation damage results from inappropriate activation of normal, physiological processes, and b) physiological manipulation of radiation response is feasible, bringing therapeutic potential. We consider the first of these in the following review.

Table 1. Signal transduction and cybernetics.

Cellular signal processing	Cybernetics
Receptors	sensors
Couplers & signal network	system control
Kinase cascades & transcription	actuators
Substrates & gene products	motors

Genome damage can mimic molecular structures capable of inducing cellular response. McClintock reported that broken chromosome ends represent one of these structures, and that response could be terminated, either by joining broken chromosome ends together or by capping the ends with telomeres [4]. Her studies suggest that the molecular structures artificially produced by radiation exposure which induce response are telomere-less chromosome ends. McClintock's 'discernible but initially unforeseen responses' of the cell to these broken chromosome ends [1] are the same cellular phenomena that accompany senescence [5], including cell cycle arrest, induction of genome lability, and programmed cell death; a term used to refer to any cell death mediated by an intracellular death program including apoptosis [6]. Severe radiation damage induces chromosome aberrations which result in cell death, both of which are observed in postsenescent crisis [5, 7–9]. Various similarities between DNA damage-induced checkpoint arrests and senescence have been reported [10, 11]. However, differences between senescence and radiation response exist. Senescence is a permanent arrest whereas in most cells radiation-induced arrest, at least at physiologically relevant doses (0–4 Gy), is transitory. Furthermore, although caffeine releases radiation- and staurosporine-induced cell cycle arrest, senescence in primary diploid fibroblasts is caffeine insensitive [12, 14 and our unpublished observations].

Radiation induces caffeine-sensitive cell cycle arrests at various positions in the cycle referred to as checkpoints [13–15]. These transitory cell cycle arrests permit repair of radiation damage before intracellular activities, asso-

ciated with progress through the cell cycle, transform the damage into an irreparable state. Are these responses induced to enhance repair? Much experimental data suggest this is not the primary purpose of these arrests. Radiation activation of *p53*, the 'guardian of the genome', causes both G1 cell cycle arrest and programmed cell death even in the same cell system [16]. Why should cells simultaneously induce repair and destruction systems? Nagasawa et al. examined the relationship between radiation-induced G2 arrest and cellular radiosensitivity in seven different cell lines [17]. They observed that in all the lines, equitoxic doses of X-rays produced equi-extensive G2 arrests. However, it was the cell lines with short arrests which were radioresistant and the cell lines with long arrests which were radiosensitive. These data argue against induced arrest acting to enhance repair. Furthermore, the duration of arrest has been shown to be independent of repair. Haploid yeast (*S. cerevisiae*) exists in either of two mating types, which can switch by means of a precisely localized double strand break (DSB). Sandell and Zakian genetically manipulated a strain of yeast so that the site of this break was located close to the end of a chromosome [18]. They then introduced DSB repair deficiency into the yeast strain. By activating the switching they produced a yeast cell with an artificially broken chromosome end which could no longer be repaired. As expected, the presence of this end induced a programmed cell cycle arrest. However, in spite of the continued presence of the break many cells eventually re-entered the cell cycle. Inability to repair the damage did not induce permanent arrest. Similar observations were made in a study of Bae et al. in human lymphoma cells [19]. Radiation-induced genome damage enhances p53 activity, this in turn upregulates expression of the *p21* gene, whose product induces G1 arrest [20], as shown in figure 1. The study of Bae et al. showed that although initiation of arrest depended on p53 activation in the presence of genome damage, the duration of arrest depended on the stability of the p21 protein, and not on the stability of the p53 protein. The duration of arrest was, therefore, independent of the state of the genome damage. The conclusion from these studies is that repair occurring during cell cycle arrest is a side-effect and not the physiological reason for arrest. The primary purpose appears to be avoidance of cellular proliferation, a phenomenon associated with the induction of cellular senescence.

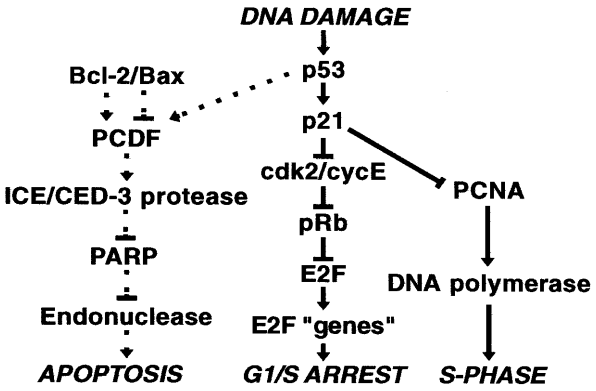

Figure 1. DNA damage-activated signal pathways. Various cellular responses to exposure to ionizing radiation are observed, including physiological cell death and cell cycle arrests. The signal pathways displayed are representative, not exclusive. Apoptosis can be induced in both a p53 dependent and independent manner. p21 does not necessarily require p53 to induce cell cycle arrest. Furthermore, many feedback controls exist and many more proteins are involved. The signal pathways should be thought of as paths within a large integrated biochemical network which governs execution of cellular response. Arrows with pointed heads indicate activatory signals, arrows with flat heads indicate inhibitory signals.

Telomeres and telomerase

Mueller [21, 22] first used the term telomere, when investigating radiation-induced chromosome aberrations in *Drosophila*. The unexpectedly low frequency of terminal deletions indicated that special structures must be present on the end of chromosomes which protect them from damage. McClintock, in an elegant series of studies in maize [4, 23], demonstrated that telomeres not only prevent chromosome ends from inappropriate fusions but also 'heal' broken ends and thereby silence signals which would otherwise induce genomic instability. McClintock created a model system for investigating the properties of single broken chromosome ends in the seeds of maize plants. Seeds carrying single broken chromosome ends displayed high levels of genetic instability (transpositions). This was observed in the triploid endosperm but was apparently 'healed' in the diploid zygote tissue. McClintock suggested that 'healing' resulted from addition of a telomere onto the broken end of a chromosome. Telomeres consist of short, highly repetitive DNA sequences. They are located at the ends of chromosomes; telomeric DNA sequences are also found at the centromeres and sometimes associated with interstitial sites of radiation-induced translocations [24, 25]. These interstitial sites are inverted repeats consisting of a short stretch of DNA surrounded by the telomere repeat sequence. In all vertebrates studied the G-rich 3′-repeat is the hexanucleotide: TTAGGG. Telomere length refers to the number of telomere repeats at the chromosome end. However, standard methods of quantifying telomere length measure both the telomeric and subtelomeric regions, referred to as the mean telomeric restriction fragment size [26]. Studies of human chromosomes indicate that the ends are complex genetic structures [27]. At the very ends are 2–20 kb of the canonical TTAGGG repeats, preceded by 1–2 kb of degenerate TTAGGG repeats. Behind these but separated from them by an approximately 200 bp unique divider sequence are two or three sets of repeat se-

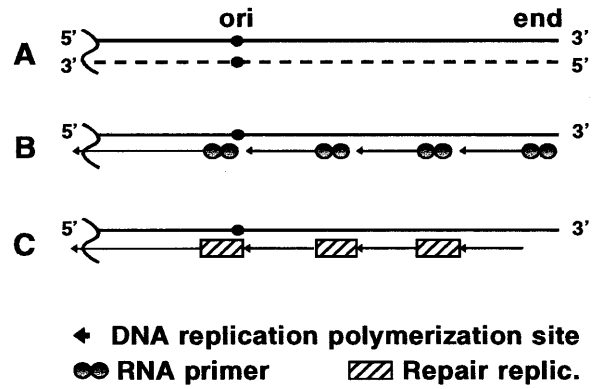

Figure 2. DNA replication at the end of a chromosome. (*A*) DNA consists of two complementary strands, a 5′–3′ and a 3′–5′. Replication starts at many sites along a chromosome, called ori (*ori*gin of replication). The DNA polymerase binds at this site but can replicate DNA only in a 5′–3′ direction and only starting from an existing DNA or RNA primer. (*B*) Additional RNA primers are placed 3′ of the ori site to initiate DNA replication. The resultant short stretches of DNA are called Okazaki fragments. (*C*) When the DNA has been replicated, the RNA primers are removed and the gaps filled by replication repair. The last gap cannot be filled because there is no DNA or primer to initiate polymerization.

quences which consist of 2–20 copies of 30–80 bp repeats. Only then comes the unique sequence DNA [28, 29]. The telomeric sequences are associated with specific proteins. A human telomere binding protein, TRF1, has been identified [30, 31]. The gene sequence was found to be homologous to the *Rap1* gene of *S. cerevisiae*, and also to the recently isolated *Taz1*$^+$ gene in *S. pombe*. Functional studies of these proteins indicate that they are able accurately to govern the length of the telomeric sequence [32–34].

Telomeres shorten at each round of cell division because of problems associated with DNA replication at the extreme ends of the chromosomes [10, 35]. DNA polymerase can move along the 5′–3′ 'leading' strand of the chromosome replicating DNA. This is not possible along the 3′–5′ 'lagging' strand. The DNA polymerase cannot move in the 3′–5′ direction, and furthermore, it requires a pre-existing 5′ DNA or RNA primer from which to initiate replication. Along the lagging strand the DNA is replicated in pieces called Okazaki fragments. Each of these fragments is replicated in a 5′–3′ direction initiated from a RNA primer. After replication, the RNA primers are removed and the short gaps remaining are filled by polymerase activity (see fig. 2). However, at the end of the chromosome, at the most distal gap, there is no pre-existing 5′ DNA for the DNA polymerase to bind to and a short length of DNA is lost. Consequently, after each division the chromosomes shorten at a rate of some tens of base pairs per cell cycle. Additional mechanisms of telomere attrition have also been reported [36]. There is a strong correlation between telomere shortening and cellular senescence [37]. In *Tetrahymena* cells with mutated telomerase RNA abnormal telomeres result and the cells display features of senescence [38]. In yeast *est1* mutants are characterized by *e*ver *s*horter *t*elomeres (*est*) and features typical of ageing and senescence [39]. Other studies have demonstrated that length of human fibroblast telomeres, like proliferative potential, is directly related to the age of the donor [40, 41]. The telomeric repeats shorten with age [42, 43] and a clear correlation between the number of divisions through which a cell has passed and the length of its telomeres exists [44]. Artificial lengthening of telomeres has been demonstrated to extend the proliferation lifespan of cell hybrids [45] and the presence of lengthened or stabilized telomeres is necessary for immortalization [46]. Chromosomes from patients with the progeric disorders Hutchinson-Gilford syndrome and Werner's syndrome (WS) have abbreviated telomeres consistent with the reduced division potential of their fibroblasts in cell culture [47–49]. The WS gene has been cloned and displays significant homology to DNA helicases [50]. The WS defect mimics the DNA damaging effects of radiation exposure, the enhanced gene mutation frequency displayed by fibroblasts from WS patients being associated with an unusually high proportion of large deletions [51–53]. Patients with *Ataxia telangiectasia*, a multi-faceted syndrome, who display abnormal sensitivity to ionizing radiation and premature ageing also have abbreviated telomeres compared with healthy individuals. The *Ataxia telangiectasia* gene has been cloned and shows significant homology with genes involved in telomere metabolism [54, 55].

Somatic cells have only a finite division potential before they senesce. However, in cells of the germ line (responsible for the perpetuation of the species) telomerase is present. This is an enzyme complex capable of maintaining telomeres at a constant length so that the cells do not senesce but are capable of unlimited division. It is a ribonucleoparticle consisting of protein subunits and an RNA molecule [56, 57]. The number of protein subunits in the telomerase complex is not yet known. However, the gene coding for the RNA molecule has been cloned and its sequence established [57a]. The RNA possesses a region of complementary homology to the telomeric repeat (fig. 3). This region acts as a template for replication of the telomeric DNA. Because telomerase synthesizes new DNA from an RNA template it is a reverse transcriptase [58]. The ability of telomerase to extend, or to hold constant, the length of the telomere over multiple cell divisions has been demonstrated to go hand-in-hand with unlimited growth potential and the development of malignancy [5]. Kim et al. demonstrated that in most of the immortalized tumour cell lines investigated (98 of 100) and in 90% of all tumours examined (90 of 101), telomerase had been reactivated [59]. To date, more than 1000 tumours have been investigated and telomerase activity

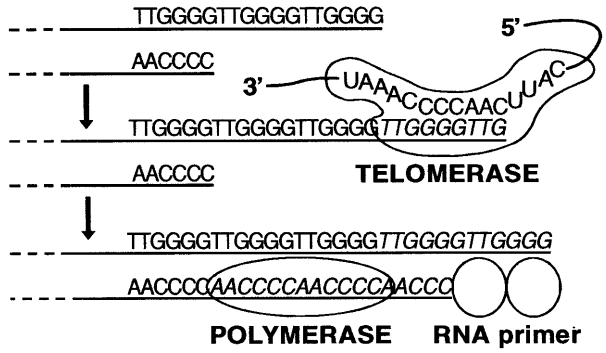

Figure 3. Telomerase extension. Telomerase, a complex of proteins and RNA, is required to lengthen telomeres. Telomerase is a reverse transcriptase. Copies of the telomeric repeat sequence are made from the RNA template within the telomerase complex. These are used to extend the longer strand of DNA. Finally, the shorter strand is replicated using an RNA primer and DNA polymerase.

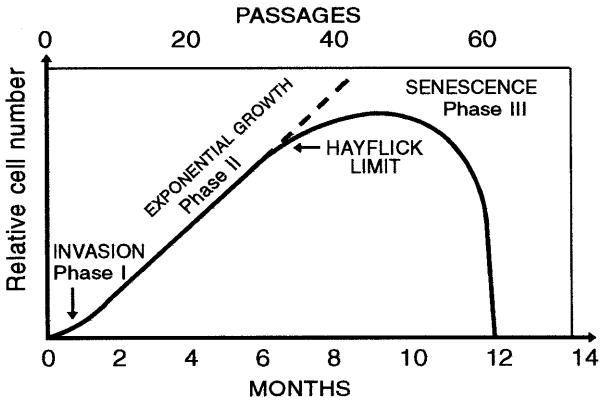

Figure 4. Growth of explanted fibroblasts. After transfer of a tissue biopsy to culture dishes, 25 fibroblasts grow out of the biopsy and onto the dish (phase I). There then follows a period of exponential growth (phase II). After a certain number of divisions growth ceases at the so-called Hayflick limit and the culture enters senescence (phase III).

has been observed in 85% [60]. The telomerase activation status of cells is a highly predictive indicator of the malignant state of a cell. However, studies by Bryan et al. temper an exclusive role of telomerase in cellular immortalization. Telomerase activity was observed in only 20 of 35 tumour-virus transformed cell lines [46]. Telomerase activity has been demonstrated in germ line and embryonic tissues [61] and in various renewal tissues including blood, skin, intestine and hair follicles [62–65]. Despite its presence in stem cells, telomeres shorten with age in human blood, endothelium and connective tissue [46–48, 66, 67]. Telomerase activity is under extensive regulation, it is repressed in differentiated and quiescent cells [62, 68], and in two different transgenic mouse models of multistage tumorigenesis, telomerase activity was observed only in late stage tumours although telomerase RNA levels were upregulated in early preneoplastic stages [69].

Senescence, ageing and tumour suppression

There are several models of cellular senescence and ageing. Among the more popular ones are those based on damage accumulation [70] or programmed senescence [71, 72]. They are not necessarily exclusive. However, weighing against a simple model of damage accumulation is both the fact that the germ line is immortal, and the fact that the so-called 'oldest old' generally appear fitter than those 20 years their junior [73]. Many new insights into the molecular biology of ageing support the programmed senescence hypothesis [72]. According to this hypothesis, after a relatively fixed or 'preprogrammed' number of divisions cells enter senescence and eventually die.

The strongest evidence for this phenomenon comes from studies of tissue explants. Tissue explants grown in culture display three growth phases. First those cells capable of growth in the extracorporeal environment invade the culture dishes. Then follows a period of exponential cell growth. Finally, cell growth terminates with the arrest of cell division, and culture senescence (fig. 4). The duration of this senescence period is variable but eventually ends in death of the cells. Programmed cell death, however, has not been observed during senescence [74]. The period of transition from exponential growth to senescence is referred to as the Hayflick limit, after the discoverer of the phenomenon. He recognized that fibroblasts from tissue explants are capable of only a limited number of cell divisions before entering senescence, and also that this depended on the age of the tissue donor [75, 76]. Thus, cells from young donors are capable of more divisions than cells from old donors. At each cell division the ends of the chromosome arms (telomeres) shorten [42, 43]. Upon reaching a critically short length a signal is generated and the cells enter senescence: M1 (mortality stage 1). If senescent cells are forced through further rounds of division their telomeres become even shorter, division ceases and programmed cell death is induced: M2 (mortality stage 2). These defense mechanisms have to be circumvented for tumours to progress from a benign state of limited growth to a malignant state of potentially unlimited growth. To evade the physiological impasse of telomeric attrition a cell must activate telomerase which prevents and can even reverse telomere shortening [62, 57a]. Although senescence based on telomeric shortening appears to set a limit to potential cell renewal, tissues have never been shown literally to run out of cells, and substantial numbers of proliferative cells can often be recovered from very old tissues [72]. However, with increasing age, increasing numbers of senescent cells are observed in tissues. These cells may age the tissues [72]. They accumulate, cannot proliferate, resist apoptotic death, have an abnormal physiology, and have an abnormal impact in the respective tissues [72, 78].

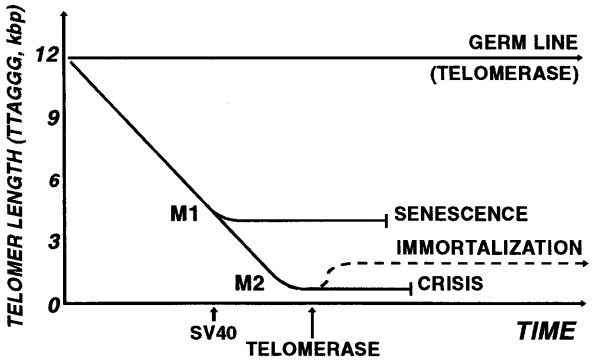

Figure 5. Cell state and telomere length. Average telomere length depends on the 'age', type, and health of a cell. With each cell division the telomeres shorten. After reaching a critical length the cells arrest and senesce. If infected with tumour viruses cells can circumvent senescence and the telomeres shorten even more. The cells eventually enter a second arrest referred to as crisis where the cells die. Cells can become immortal at any stage if their telomerase is activated. In transformed cells this leads to malignancy. The germ line has a temporarily-controlled, constitutive telomerase activity which confers immortality to a species.

When cells enter senescence they take on an altered morphology [79]. The cytoplasm of the senescent cells flattens out and becomes more circular, taking on an extended saucer-like appearance. This indicates that extensive remodelling of the cytoplasm has taken place. Senescent cells arrest in the G1 phase of the cell cycle and the CDK/CYCLIN complexes which drive the cell cycle are expressed abnormally [80, 81]. Furthermore, although the senescent cells are eventually destined to die, they are resistant to apoptosis [82]. Senescence arrest can be circumvented by the action of tumour viruses such as SV40, human papilloma viruses, etc. The viruses produce proteins such as large T antigen (SV40), or E6 and E7 (human papilloma viruses), which bind to the cell cycle regulatory proteins p53 and Rb [83, 84]. This binding prevents the normal inhibitory function of these proteins, permitting signals which promote cell growth to stimulate cell division. Postsenescent cells display a spindle morphology characteristic of non-senescent cells and they continue to grow for a limited number of divisions resulting in the cells' telomeres becoming even shorter. This extreme shortening is associated with chromosome instability [40, 85]. The cells then enter a second irreversible arrest, referred to as crisis or M2, and die. However, a few cells avoid crisis by overcoming telomere attrition either by activation of telomerase or recombination mechanisms [86]. Those few cells which sidestep the second arrest become immortal, having the potential to proliferate indefinitely (fig. 5).

A two-stage molecular senescence mechanism is recognized based on the cdk inhibitors p21 and p16 [87]. As human diploid fibroblasts approach senescence, cellular p21 protein expression is induced and the cells arrest in G1. Subsequently, cellular p16 protein expression is induced and p21 levels decline. It appears that p21 is involved in the initial induction of senescent G1 arrest and p16 for its extended maintenance. The *p21* gene can be transcriptionally activated by the *p53* gene product. Expression of mutant *p53* in presenescent cultures prevents senescence arrest and results in supernumerary cell divisions and culture crisis where a second round of arrest and cell death occurs [88]. Although cellular p21 proteins levels fall during senescence [87], during supernumerary divisions the p21 levels rise again reaching a maximum at crisis, only to fall if the cells become immortalized [88–90]. p16 prevents CDK4 and CDK6 from phosphorylating Rb, which continues to bind E2F preventing transcription of genes required for cell cycle progression [87]. When human diploid fibroblasts enter senescence antisense-*Rb* oligomers extend the lifespan of the cultures by an additional ten cell divisions before crisis. Although the cells are refractory to antisense-*p53* oligomers, both oligomers together extend the lifespan of the cultures by an additional 20 cell divisions [91]. The expression of oncogenic *ras* in primary human and rodent cells has been shown to result in a G1 arrest phenotypically indistinguishable from cellular senescence, and sensitive to inactivation of p53 or the CDK inhibitor p16, which confirms that senescence can be induced at any time, either immediately by oncogenic activation or eventually by telomere attrition [92]. Cellular immortality requires inactivation of the P53-dependent senescence mechanism and inactivation of the P16-dependent senescence mechanism and prevention of telomere attrition [93].

When telomere attrition reaches a critical length, about 4 kb, cells enter G1 arrest and senesce [94]. It is thought that a signal associated with telomere attrition activates the p53 and Rb proteins which promote the senescent state [95]. When cells are irradiated they act in a similar manner. The chromosome breaks induce a signal causing the cell cycle machinery to activate cell cycle arrest. Arrest involves the p53 protein, which promotes the activity of p21 [91, 96, 97]. p21 inhibits the CDK/CYCLIN complexes. The G1 arrest signal induced by DSB after radiation damage is p53 dependent and partially p21 dependent [97, 98]. In addition, the abnormal cyclin expression observed in senescent cells is also parallelled by the repression of cyclin B1 gene expression observed in HeLa cells during radiation-induced G2 arrest [99]. However, there is little evidence of p16 or Rb being induced during the response to DNA damage [5, 100], and although caffeine can release the various cell cycle arrests induced by ionizing radiation and the protein kinase inhibitor staurosporine, it cannot release senescence arrest [14 and our unpublished results]. We attribute this to the more permanent form of senescence arrest requiring both p21 and p16. The telomeres of those cells forced to divide after entering senescence continue to shorten. When the average

telomere length is only 1 kb there is a high probability that at least one of the chromosome ends is stripped of all its TTAGGG sequences [35]. It is this 'naked end' which is thought to induce crisis and cell death. Cytotoxicity in crisis appears to be caused by two conflicting signals: a signal via p53 telling the cell to stop division and a signal, caused by the viral oncogene, telling the cell to continue division [72]. Radiation causes a similar signal conflict situation in transformed cells. These cells already possess abnormal proliferation signals and together with the radiation-induced arrest signals apoptosis is induced. If the *p53* gene is mutated in these cells apoptosis is not induced and the cells become resistant to radiation treatment [101]. This enhanced survival can compromise radiation therapy of tumours. p53 acts in a p21-independent manner to induce apoptosis, but in a p21-dependent manner to induce cell cycle arrests. However, functional p53 is not obligatory for cellular response to genotoxic damage [97, 98].

Conclusions

If transformed cells do not immortalize they tend to form benign tumours. Cellular senescence, by preventing immortalization, is a physiological defense against malignant tumours. Senescence is probably the most important tumour suppressor mechanism and may be the primary defense against carcinogenesis [102]. Ageing is a natural consequence and the price paid for this extremely efficient anti-oncogenesis mechanism [78] and if it were possible to reset the ageing 'clock' via selective telomerase activation, specifically in nontumorigenic cells, increased lifespan might be feasible. The cell is programmed to respond to telomere attrition and telomere-less chromosome ends by inducing cell cycle arrests or programmed cell death. Both of these responses prevent cellular proliferation. At the molecular level many of the same proteins shown to regulate programmed senescence and crisis are also involved in radiation response. Therefore, many of the programmed cellular responses to ionizing radiation exposure, including the various cell cycle arrests and programmed cell death, result from inappropriate induction of pre-existing cellular senescence mechanisms. However, the potentially modifying influences of DSB-associated telomeric sequences and the different sensitivities to caffeine indicate that some differences between the two phenomena exist. Neither does ionizing radiation cause tissues to age [103], probably because the arrests it induces are transitory and not permanent, unlike those caused in senescent cells. Telomere length in immortal tissues (e.g. germ line or tumour cells) is preserved because of the activity of telomerase. No existing tumour marker or oncogene is as frequently observed in as many different tumours types as ectopic telomerase activity [59]. Cell immortalization requiring abnormal telomerase activity appears to be an almost universal step in carcinogenesis. Telomerase activity has high prognostic value and could provide the clinician with new alternative and supplementary therapies. An intensive search for telomerase inhibitors which might re-initiate telomere attrition and restore finite division potential to tumour cells, converting malignant into benign tumours [10, 104] is currently underway. One fascinating example of this is the 'curing' of HeLa cells by transfection with antisense human telomerase RNA [57a].

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