Pergamon

European Journal of Cancer Vol. 33, No. 5, pp. 710–715, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00090-7

Multiple Pathways to Cellular Senescence: Role of Telomerase Repressors

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Telomeres progressively shorten with age in somatic cells in culture and *in vivo* because DNA replication results in the loss of sequences at the 5′ ends of double-stranded DNA. Whereas somatic cells do not express the enzyme, telomerase, which adds repeated telomere sequences to chromosome ends, telomerase activity is detected in immortalised and tumour cells *in vitro* and in primary tumour tissues. This represents an important difference between normal cells and cancer cells, suggesting that telomere shortening causes cellular senescence. Hybrids between immortal cells and normal cells senesce, indicating that immortal cells have lost, mutated or inactivated genes that are required for the programme of senescence in normal cells. Genes involved in the senescence programme have been mapped to over ten different genetic loci using microcell fusion to introduce human chromosomes and restore the senescence programme. Multiple pathways of cellular senescence have also been demonstrated by chromosome transfer, indicating that the functions of the mapped senescence genes are probably different. One possibility is that one or more of these senescence genes may suppress telomerase activity in immortal cells, resulting in telomere shortening and cellular senescence. To test this hypothesis, telomerase activity and the length of terminal restriction fragments (TRFs) have been examined in microcell hybrids. Re-introduction of a normal chromosome 3 into the renal cell carcinoma cell line RCC23, which has the short arm of chromosome 3, restored cellular senescence. The loss of indefinite growth potential was associated with the loss of telomerase activity and shortening of telomeres in the RCC cells containing the introduced chromosome 3. However, microcell hybrids that escaped from senescence and microcell hybrids with an introduced chromosome 7 or 11 maintained telomere lengths and telomerase activity similar to the parental RCC23. Thus, restoration of cellular senescence by chromosome 3 is associated with repression of telomerase function in RCC cells. This evidence suggests that telomerase suppression is one of several pathways involved in immortalisation.

Key words: cellular senescence, telomere, telomerase, multiple pathways, microcell-mediated chromosome transfer

Eur J Cancer, Vol. 33, No. 5, pp. 710–715, 1997

INTRODUCTION

A SINGLE chromosome can be introduced into a variety of immortal or tumour cells *in vitro* via microcell-mediated chromosome transfer [1–3]. Alteration of cellular phenotypes in microcell hybrids by transfer of specific chromosomes has allowed speculation about the functions of the gene(s) on the transferred chromosome [3–5]. Thus, this method has been used for identification of chromosomes carrying tumour suppressor genes, cellular senescence genes, and genes responsible for recessive-inheritable diseases [3, 6, 7]. Several chromosomes that suppress various transformed phenotypes in a number of tumour cell lines have been identified with this method. Four types of suppression have been identified: the induction of cellular senescence, the suppression of *in vitro* transformed properties and tumorigenicity, the suppression of tumorigenicity alone, and the supression of metastasis alone [8, 9].

Normal cells *in vitro* and *in vivo* have a limited lifespan, whereas tumour cells can often grow indefinitely [10]. At

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the end of their proliferative lifespan in culture, normal cells exhibit morphological changes, become enlarged, and cease proliferation, a process termed cellular senescence [10–12]. Escape from cellular senescence predisposes a cell to neoplastic conversion [13–15]. It has been proposed that cellular senescence is controlled by genes which are activated or have functions that become evident at the end of the proliferative lifespan of the cell [6, 12]. According to this hypothesis, a family of senescence genes exists, and immortalisation occurs due to defects in these genes that allow cells to escape the programme of senescence. Hybrids of normal and tumour cells generally senesce, indicating that normal cells can correct defects in the senescence programme of tumour cells [16].

Over ten senescence genes on human chromosomes have been mapped using microcell-mediated chromosome transfer [5, 6, 17–24]. It has been recently proposed that telomeres, which shorten with each cell division, act as a 'molecular clock' defining limited proliferative potential [25, 26]. Telomerase, a ribonucleoprotein which extends shortened chromosomal ends with repeated TTAGGG sequences, is re-expressed in most tumour and immortal cells, while most normal somatic cells have no detectable activity resulting in telomere shortening [26, 28]. Therefore, normal somatic cells are considered to have a genetic mechanism for repressing telomerase activity, while immortal tumour cells are considered to have mutated, inactivated or lost putative repressor gene(s). Introduction of normal human chromosome into various immortal cell lines restores cellular senescence either with or without repression of telomerase activity, suggesting that telomerase repression is involved in one of several pathways regulating cellular senescence.

MAPPING OF PUTATIVE SENESCENCE GENE

Using a mouse A9-human chromosome library consisting of cell clones with an individual pSV2neo-tagged human chromosome, different chromosomes have been independently transferred to various immortal cells via microcell fusion, and selected for G418-resistant cells. Over ten senescence genes have now been mapped to chromosomes (Table 1). For example, when chromosome 1 was introduced into HHUA cells, 70 of 92 clones (76%) senesced [5]. Surprisingly, 31 of 97 clones (32%) with an introduced chromosome 18 also senesced. However, none senesced among 59 clones selected after transfer of either chromosome 6, 9, 11 or 19. Thus, the HHUA microcell hybrids with chromosome 1 or 18 exhibited two types of morphology; some of the cells consisted of cells with polygonal cells similar to the parental cells and the other clones were composed of flat, senescent-like cells. The cells with a flat morphology also had a decreased growth rate; their doubling time was 46–52 h versus 24–30 h for the parental HHUA. Many of these clones ceased proliferating and senesced. The clones that did not senesce were overgrown by a subpopulation of cells that re-exhibited the parental cell morphology and growth rate. The doubling times of the non-morphologically altered clones were the same as the parental cells or microcell hybrids with other chromosomes.

Karyotypic analysis has also been performed on the microcell hybrids. Karyotypic analysis of the flat, senescent-like cells showed an extra intact copy of chromosome 1 in the

Table 1. Induction of cellular senescence by normal chromosome and telomerase activity

Transferred chromosome	Cell lines	[Ref.]	Telomerase activity†
#1	TE85 (Osteosarcoma)	[23]	+ → +
	143 B TK$^-$ (Ki-Ras$^+$-transformed TE85)	[23]	
	CMV-Mj-HEL-1 (CMV-transformed lung fibroblasts)	[23]	
	HHUA (uterine endometrial carcinoma)	[24]	
	Ishikawa (uterine endometrial carcinoma)	*	
	10W (chemically-induced Syrian hamster fibroblasts)	[6]	
	B16-F10 (mouse melanoma)	*	+ → +
#2	SiHa (cervical carcinoma)	[22]	+ → +
	B16-F10 (mouse melanoma)	*	+ → +
#3	RCC23 (renal cell carcinoma)	[42]	+ → −
	KC12 (RCC in VHL)	*	+ → −
	TS1 (lung adenocarcinoma)	*	+ → +
#4	HeLa (cervical carcinoma)	[17]	
	J82 (bladder cancer)	[17]	
	T98G (glioblastoma)	[17]	
#6	SV/HF (SV40-transformed human fibroblasts)	[20]	
#7	SUSM-1 (human transformed fibroblasts)	[19]	
	KMST-6 (human transformed fibroblasts)	[19]	
	CC1 (choriocarcinoma)	*	+ → +
#10	HMc-Li7 (hepatocellular carcinoma)	*	+ → −
#11	RD (rhabdomyosarcoma)	[18]	
	H15 (bladder cancer)	*	
#18	HHUA (uterine endometrial carcinoma)	[24]	
X	HocB (ovarian carcinoma)	*	
	ELCO (breast carcinoma)	*	

*Unpublished data by the authors.

†+, telomerase activity; −, no telomerase activity. The arrow shows the change in activity with the cell line following transfer of the normal chromosome indicated.

majority of hybrid clones. In contrast, none of the eight clones with increased growth and normal morphology had an intact copy of the introduced chromosome. Likewise, senescent clones resulting from microcell transfer of chromosome 18 had an intact, extra copy of chromosome 18 in five of five clones, whereas chromosomes 6, 9, 11 or 19 retained intact copies of the introduced chromosomes in most clones analysed.

This study did not permit definition of the regions on chromosomes 1 and 18 involved in the induction of cellular senescence. Previous studies have mapped the location of cellular senescence genes on chromosome 1 to two regions [30]. The regional mapping of the gene on chromosome 18 will require additional studies and isolation of more hybrids that escape senescence.

MULTIPLE PATHWAYS TO CELLULAR SENESCENCE

The senescence programme could be activated by a single pathway and immortal cells could arise due to mutations in any of the genes that encode proteins required in this pathway. An alternative hypothesis is that the senescence programme is activated by multiple, independent pathways (Figure 1). As described, introduction of either of two different chromosomes induces senescence in the same immortal cell line. Additional evidence for multiple pathways for inducing cellular senescence is that B16-F10 senesces following introduction of human chromosome 1 and 2 (M. Oshimura, Department of Molecular and Cell Genetics, Tottori University, Japan). This finding implies that multiple pathways of senescence exist and that immortal cells arise due to defects or mutations in genes in each of the pathways. Mutations that affect only a single pathway would not result in cells that were immortal, but the cells might have an extended lifespan. For example, SV40 infection (which inactivates pRb and p53 proteins) results in an extended lifespan but not immortalisation of infected cells [31, 32]. Additional genetic changes, possibly loss of chromosome 6, are required for immortalisation of SV40-infected human cells [33, 34]. Re-introduction of a normal chromosome 6 results in senescence of SV40 immortal cells [20]. Antisense downregulation of pRb and p53 mRNAs also results in extension of the lifespan of human cells without immortalisation [35]. Based on the results with SV40 infection, the most likely number of pathways for human fibroblast immortalisation is at least three, one involving pRb, one involving p53, and one involving an unknown gene, possibly on chromosome 6 [33]. The 'multiple pathways to senescence' hypothesis is consistent with the multistep nature of chemically induced immortalisation [36, 37]. Furthermore, the inability to assign certain immortal cells to a single complementation group is also explained by this hypothesis [38, 39].

EVIDENCE FOR TELOMERASE REPRESSOR GENES

Activation of senescence pathways is likely to involve complex regulatory mechanisms. The correlation of the proliferative potential of a cell with cumulative population doublings, independent of chronological time, suggests that a

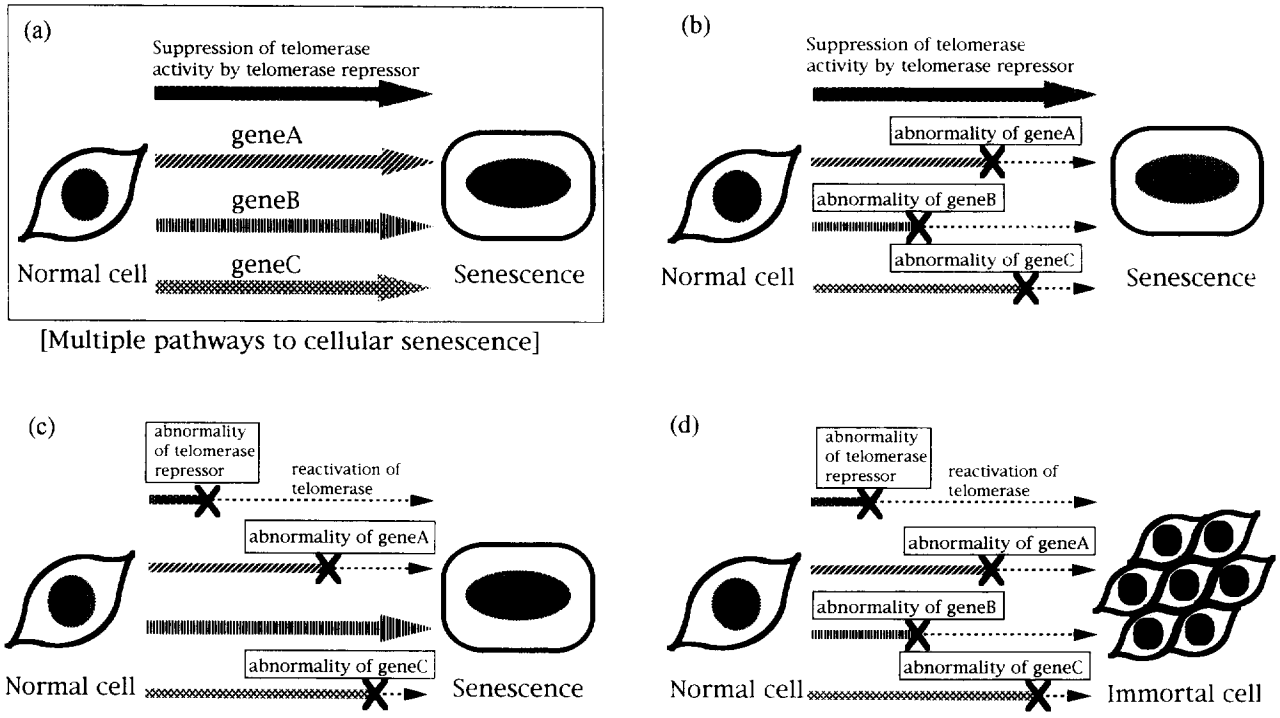

Figure 1. A model for multiple pathways to cellular senescence. (a) In normal cells, telomerase activity is suppressed by the telomerase repressor. The other pathways function normally, resulting in senescence. (b) Although several pathways are abnormal (A, B and C senescence genes are lost, mutated or inactivated in this case), cells senesce because of the repression of telomerase activity. (c) There is telomerase activity because of loss, mutation or inactivation of telomerase repressors. However, since one of pathways is functioning normally, cells senesce. (d) All the pathways are abnormal with telomerase activity resulting in cellular immortalisation.

counting mechanism(s) exists to record cellular divisions. There are some measurable parameters that correlate with the decreasing replicative potential of human fibroblasts. One of these is chromosome telomere length. Telomeres are terminal eukaryotic chromosome sequences consisting of short (2–12 base pairs) nucleotide repeats and associated proteins [40]. Telomere length has been reported to shorten in normal cells as replicative capacity decreases [25], and there is a strong correlation between human fibroblast telomere length and remaining *in vitro* replicative capacity [41]. As observed, a decrease in average telomere length following restoration of limited proliferative potential in an immortal renal carcinoma cell line by introduction of a normal human chromosome 3, provides further evidence of a relationship between telomere length and cellular proliferative capacity.

RCC23 is a non-tumorigenic human renal cell carcinoma cell line, established from the primary tumour tissue of a 56-year-old female with non-papillary carcinoma stage III. Karyotype analysis showed loss of the short arm of chromosome 3 due to an unbalanced translocation between chromosomes 3 (breakpoint at p11) and 8 (breakpoint at q11). RFLP analysis also revealed that the renal cell carcinoma cell line RCC23 exhibited allele loss on the short arm of chromosome 3 [42].

In microcell hybrids with an introduced chromosome 3, saturation densities were reduced, and their population doubling times were prolonged compared with the parental cell line (Figure 2). The microcell hybrids were flat in shape and clearly different from the recipient RCC23 cells. Cells from three clones senesced after 23–43 population doublings. One of the microcell hybrid clones regained a similar morphology, growth rate and saturation density to those of the immortal RCC23 cells. Chromosome and RFLP analyses revealed that the MC#3-8R had lost the introduced chromosome 3. Chromosomes 7 or 11 were also introduced to RCC23 cells: all of these clones showed similar biological and morphological features of the parental RCC23 cells [42].

TRF (terminal restriction fragment) lengths were determined by hybridisation of the $(TTAGGG)_4$ probe to IInfI-digested DNAs from cultured normal kidney cells, RCC23, RCC23 microcell hybrids containing chromosome 3 (MC#3), chromosome 7 (MC#7) or chromosome 11 (MC#11) and from the revertant clone, MC#3-8R. The peak TRF values were 7.3 kb and 5.8 kb for cultured normal kidney cells and RCC23 cells, respectively. Although the normal kidney cells and RCC23 DNAs were not obtained from the same individual, their TRF lengths were compatible with results previously reported by others [43], indicating that telomere shortening is associated with malignant transformation of RCC23 cells. The MC#3 clones showed a significant reduction in *in vitro* growth rate with morphological alterations, and senesced after several months of culture (23–43 population doublings). All these clones showed a reduction in the TRF lengths, when compared with parental RCC23 cells: 2.9–3.1 kb versus 5.8 kb. The difference in TRF lengths between RCC23 cells and MC#3 clones was 2.7–2.9 kb. If the mean reduction rate in MC#3 clones is assumed to be the rate of telomere reduction of normal cells *in vitro* (65 bp/generation) [26], the calculated number of generations required for the observed telomere shortening (2.8 kb) is 43, which is consistent with the population doublings of the microcell hybrids when TRFs were examined just prior to senescence. After several passages in culture, one clone MC#3-8 lost the introduced chromosome 3, and regained similar growth properties and morphology to those of the parental RCC23 cells, and continued to proliferate even beyond 100 population doublings (MC#3-BR). Interestingly, the telomere length in the MC#3-8R cells was longer than that of the original MC#3-8 cells: 2.9 kb versus 4.3 kb. The TRF lengths in MC#7 and MC#11, which did not senesce, were similar to the parental RCC23 cells.

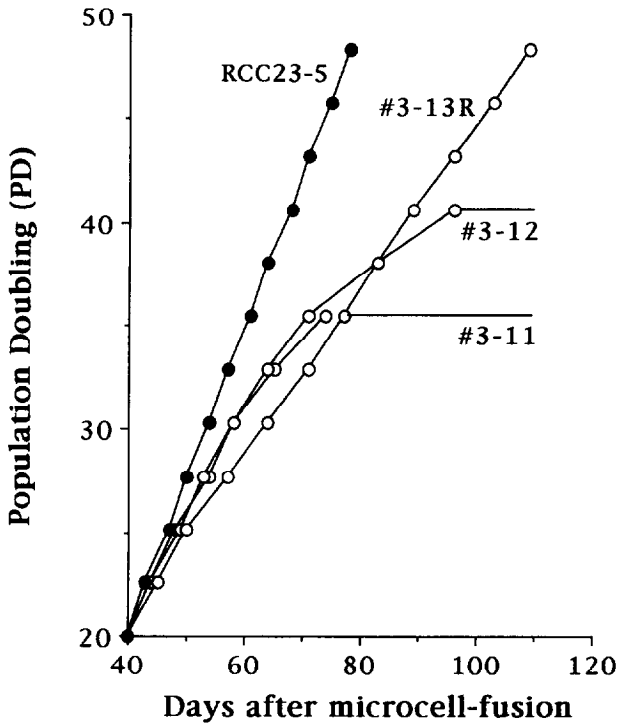

Figure 2. Growth curve of RCC23-5 subclone and three microcell hybrid clones with the introduction of chromosome 3, i.e. #3-11, -12 and -13R. To determine the population doubling, cells were plated in a 100 mm dish at a density of 1.5×10^5 cells for each passage, when cell density reached 80% confluence [42].

To examine if telomere reduction was due to loss of telomerase activity in cells, its activity was measured by the primer extension TRAP assay [44], in which telomerase synthesises telomeric repeats on to oligonucleotide primers [25]. MC#3 clones examined showed markedly reduced telomerase activity (less than 1/10 of RCC23 cells), whereas MC#3-8R cells and other microcell hybrids with the introduced chromosome 7 or 11 maintained a similar activity level to that in the parental RCC23 cells. It is a possibility that telomerase-negative cells pre-existed in the original RCC23 cell population, and they were selected. However, all ten subclones, isolated from RCC23 cells, were positive for telomerase activity. Furthermore, similar effects of chromosome 3 were observed in an additional experiment using a freshly isolated subclone (RCC23-5), which was positive for telomerase activity (Figure 2). Those MC#3 microcell hybrid clones were isolated and each proliferated slower than the parental RCC23-5 cells. Two clones (MC#3-11 and -12) stopped dividing at 36 and 42 population doublings after the introduction of chromosome 3, whereas the remaining clone (MC#3-13) continued growing

like parental cells even after 70 or more population doublings. Chromosome analyses revealed that an intact, transferred chromosome 3 was present in MC#3-11 and -12 cells, whereas chromosome 3 was lost in MC#3-13 cells. Therefore, this clone is likely to be a revertant (MC#3-13R). Telomerase activities of the clones were examined at 25 and 35 population doublings: the parental RCC23-5 cells and clone MC#3-13R cells were positive for telomerase activity, whereas clones MC#3-11 and MC#3-12 were negative at both passages. While the TRF lengths were maintained in the parental RCC23-5 and clone MC#3-13R cells, the TRF lengths were reduced as a function of passage in clones MC#3-11 and -12. Mixing of telomerase-positive and -negative extracts from RCC23 and MC#3-2, or MC#3-4 cells, excludes the possibility of the presence of some inhibitory factors for telomere extension in the telomerase-negative clones.

CONCLUSION

The link between replicative senescence, immortalisation and the shortening of telomeres was documented by Harley and coworkers [45]. In somatic cells, telomerase activity is repressed and telomeres progressively shorten with each cell division [28]. Cells may stop dividing when the telomere reaches a critical length in a proliferative cell, although little is known about the mechanisms linking telomere loss to cessation of cell proliferation at the M1 (mortality 1) stage [29, 27]. Immortalisation of cells is associated with activation of telomerase activity at or near crisis of the M2 (mortality 2) stage, and immortal tumour cells can maintain telomere length [9, 45]. We have also observed a strong association of telomere loss with the restoration of the cellular senescence programme in tumour cells. Because senescence was induced by the introduction of chromosome 3, but not by chromosome 7 or 11, our findings indicate the presence of a gene(s) on human chromosome 3 whose gene product can repress telomerase activity. However, the gene for telomerase repression may or may not be the putative tumour suppressor gene on chromosome 3 thought to be important for renal cell carcinomas.

Normal somatic cells in culture generally senesce whereas tumour-derived cells are often, but not without exception, immortal and grow indefinitely [46, 47]. As shown by somatic genetic studies, for cells to escape the senescence programme, normal genes must be lost or inactivated [17–24]. For example, the introduction of specific chromosome(s) by microcell-mediated chromosome transfer has been shown to induce senescence of human and rodent tumour and transformed cells by this method. As described, two different normal chromosomes induce senescence in the same endometrial carcinoma cell line, which suggests that multiple pathways to senescence are inactivated in this cell line [5]. Thus, this hypothesis has implications for the mechanisms of cellular senescence and its role in carcinogenesis. Because chromosome 3 could not induce cellular senescence in a human cervical carcinoma cell line, which is strongly positive for telomerase activity, all tumour cells may not be defective for the same gene controlling telomerase activity. According to the multiple pathways model, the senescence programme is activated by multiple, independent pathways and immortal cells arise due to defects or mutations in genes in each of the pathways [5]. Our data are consistent with the hypothesis that the telomerase function is one of the pathways involved in immortalisation and each pathway may involve multiple genes (Figure 2).

Telomerase activity has been detected in extracts from immortal cell lines, and also in the tumour tissue, but not normal cell strains [26, 48, 47] or normal tissue adjacent to an ovarian carcinoma [50]. Kim and associates have recently examined both normal (mortal) cell strains and immortal cell lines, in addition to tumour and normal tissue biopsies, for telomerase activity using a novel polymerase chain reaction-based assay. In this study, 98 of 100 immortal cell lines, 90 of 101 malignant tumour biopsies, and all normal germline tissue biopsies were reported to harbour telomerase activity. In contrast, no activity was found in 22 normal cell strains or 50 normal or benign, somatic tissue biopsies [28]. The correlation of both advanced stage malignancy and *in vitro* cellular immortality with telomerase activity provides additional support for the hypothesis that cellular immortalisation is required for extended tumour cell proliferation *in vivo*.

One hypothesis to explain how telomere shortening could affect gene expression is that the shortening of telomere sequence through cellular divisions represses or upregulates the expression of regulatory genes by moving them into or out of transcriptionally inactive heterochromatin regions [45]. Experiments to address directly the role that telomere length plays in the regulation of cellular lifespan await the cloning of the telomerase gene(s) and novel senescence genes that are modulators of telomerase activity or expression.

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