ADVANCES IN CELL AGING AND GERONTOLOGY

Telomerase, Aging and Disease

Mark P. Mattson
editor

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PREFACE

From the fertilized egg in the beginning of development, and throughout adult life until the last breath is taken, cells in all tissues are in a dynamic state as they may divide, carry out their functions and adapt to environmental demands. In some tissues, cells have only a relatively short lifespan during which they perform their functions. It is critical that these cells die and then are removed from the tissue without adversely affecting neighboring cells. On the other hand, cells in other tissues, such as neurons, must remain viable and in communication with their neighbors throughout life in order for that tissue to carryout its various functions. While the molecular and cellular mechanisms that modulate cell division, differentiated functions and death are complex, emerging data suggest a particularly important role for the telomerase enzyme in development and aging. Accordingly, telomerase is also increasingly implicated in the pathogenesis of a variety of age-related diseases ranging from cancer to neurodegenerative disorders.

The present volume of *Advances in Cell. Aging and Gerontology* critically reviews the rapidly advancing area of telomerase research with a focus at the molecular and cellular levels. The clearly established function of telomerase is to maintain chromosome ends during successive rounds of cell division by adding a six base DNA repeat on to the telomeric ends of chromosomes. As presented in the chapters of this volume, the mechanisms that regulate telomerase expression and activity are complex. Moreover, emerging data suggest additional roles for telomerase in the regulation of cell differentiation and survival. This volume begins with an Introductory Forward by Jerry Shay and Woody Wright that raises fundamental issues concerning the roles of telomerase in aging, and the interrelationships between genomic instability, cell proliferation, aging and cancer.

The next two chapters by Klapper and co-workers and Liu detail the complex molecular interactions required for telomerase reverse transcriptase activity, and how this machinery is regulated at the transcriptional and post-transcriptional levels. An increasing number of proteins that interact with either the catalytic subunit of telomerase (TERT) or telomeres, and their relationship to DNA remodeling and chromosome maintenance are presented. Interestingly, a number of second messenger pathways are being identified that can modulate telomerase activity and/or TERT expression, some of which have interesting implications for development, aging and disease. The mechanisms whereby telomerase regulates the cell cycle on the one hand, and may be modulated by the cell cycle on the other hand, are detailed by Tej Pandita in a chapter on Telomerase and the Cell. Cycle. Lynne Elmore and Shawn Holt provide a more detailed consideration of the role of telomerase in regulation of cell proliferation and cancer with a focus on the transition to immortality. Martha Stampfer and Paul Yaswen present an intriguing hypothesis for the mechanisms controlling the transformation of cells and reactivation of telomerase during this process.

A mode of programmed cell death called apoptosis is increasingly recognized as playing pivotal roles in normal development and in many different disease conditions.

Recent evidence that telomerase, and TERT in particular, play important roles in preventing apoptosis is presented in a chapter written by my colleagues and me at the National Institute on Aging. By suppressing apoptotic biochemical cascades, TERT may play important roles in modulating developmental cell death and cell death in pathological conditions such as neurodegenerative disorders and ischemic injury. A better understanding of the anti-apoptotic function of TERT may lead to novel therapeutic strategies for disorders that involve abnormal apoptosis. As is the case in most tissues, telomerase is expressed during early development of the nervous system. In an additional chapter, my colleagues and I consider the evidence suggesting that telomerase plays important roles in regulating the proliferation, differentiation and survival of cells during brain development.

Maria Blasco has been a leader in the development and characterization of telomerase knockout mice. She contributes a chapter to the present volume detailing the phenotypic alterations in mice lacking the RNA component of telomerase. These mice are providing an invaluable model for understanding the roles of telomerase in cancer and aging, and are also likely to contribute greatly to our understanding of a variety of age-related diseases. Tissue-specific abnormalities in these mice suggest complex roles for telomerase in maintenance of organ function and in responses to environmental challenges.

In the final chapter, Len Hayflick provides a provocative commentary presenting his views of the history of telomerase research in relation to aging, and considers the future directions in this field. It is expected that this quite comprehensive volume will provide a valuable resource for graduate students and postdocs in the telomerase field and for established investigators in other fields who are beginning to study telomerase in their particular research program. With an increasing number of proteins being brought into the fold of telomerase research (e.g., DNA damage and repair response proteins, heat-shock proteins, and proteins in various signal transduction cascades) many new scientists are beginning to study this enzyme from novel vantage points. I expect that many surprises will emerge from these studies in the not too distant future.

Mark P. Mattson

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CHAPTER 1

FORWARD: AGING AND CANCER: ARE TELOMERES AND TELOMERASE THE CONNECTION?

JERRY W. SHAY and WOODRING E. WRIGHT

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This volume of "Advances in Cell Aging and Gerontology" focuses on the role of telomerase as it relates to aging and cancer. Since most aspects of this rapidly expanding field are reviewed in the accompanying articles, the intent of this introductory chapter is to provide a brief background and some terminology in the field as well as to give a perspective of where the telomerase field has progressed, some recent misconceptions that have arisen during the previous few years, and what we believe will occur in the future.

Telomerase, Aging and Disease. Ed. by M. P. Mattson and T. K. Pandita. 1 — 13

1. Introduction/background

1.1. Telomeres

Telomeres are the repetitive DNA sequences at the end of all linear chromosomes [1, 2]. In humans there are 46 chromosomes and thus 92 telomere ends. Each telomere contains thousands of repeats of the six nucleotide sequence, TTAGGG [3, 4]. The mechanisms of DNA replication in linear chromosomes is different for each of the two strands (called leading and lagging strands). The lagging strand is made as a series of discrete fragments, each requiring a new RNA primer to initiate synthesis. The DNA between the last RNA priming event and the end of the chromosome cannot be replicated because there is no DNA beyond the end to which the next RNA primer can anneal, thus this gap cannot be filled in (this is referred to as the "end replication problem") [5]. Since one strand cannot copy its end, telomere shortening occurs during progressive cell divisions. The shortened telomeres are inherited by daughter cells and the process repeats itself in subsequent divisions [6].

1.2. Replicative aging

In contrast to tumor cells, which can divide forever (are "immortal"), normal human cells have a limited capacity to proliferate (are "mortal") [7]. In general, cells cultured from a fetus divide more times in culture than those from a child, which in turn divide more times than those from an adult. The length of the telomeres decreases both as a function of donor age and with the number of times a cell has divided in culture [8]. There appear to be two mechanisms responsible for the proliferative failure of normal cells [9, 10]. The first, M1 (Mortality stage 1), occurs when there are still at least several thousand base pairs of telomeric sequences left at the end of most of the chromosomes. M1 may be induced by a DNA damage signal produced by one or a few of the 92 telomeres that have particularly short telomeres [11]. The M1 mechanism causes a growth arrest mediated primarily by the tumor suppressor genes p53 [12]. There is also some evidence for p16/pRB function in cellular senescence but this may be due in part to inadequate culture conditions (described in a later Section). However, if the actions of p53 and p16/pRB are blocked, either by mutation or by binding to viral oncoproteins, then cells can continue to divide and telomeres progressively shorten until the M2 (Mortality stage 2) mechanism is induced. M2 represents the physiological result of critically short telomeres when cells are no longer able to protect the ends of the chromosomes, so that end-degradation and end-to-end fusion occurs causing genomic instability and cell death. In cultured cells, a focus of immortal cells occasionally arises. In most cases, these cells have reactivated the expression of telomerase, which is able to repair and maintain the telomeres.

1.3. Telomerase

Telomere terminal transferase or telomerase (TEE-LÓM-ER-ACE) is a ribonucleoprotein reverse transcriptase enzyme (composed of both RNA and proteins) [13-21].

It uses its internal RNA component (complementary to the telomeric single stranded overhang) as a template in order to synthesize telomeric DNA (TTAGGG)n, directly onto the ends of chromosomes. After adding six bases, the enzyme pauses while it repositions (translocates) the template RNA for the synthesis of the next six base pair repeat (e.g. telomerase is processive). This extension of the 3' DNA template eventually permits additional replication of the 5' end of the lagging strand, thus compensating for the end-replication problem. The enzyme is expressed in embryonic cells and in adult male germline cells [22], but is undetectable in most normal somatic cells except for proliferative cells of renewal tissues (e.g. hematopoietic stem cells, activated lymphocytes, basal cells of the epidermis, proliferative endometrium, and intestinal crypt cells). In normal somatic cells, even including stem-like cells expressing telomerase, progressive telomere shortening is observed, eventually leading to greatly shortened telomeres and to a limited ability to continue to divide.

1.4. Preventing cellular aging

While there have been many studies indicating a correlation between telomere shortening and proliferative failure of human cells, the proof that it is causal was demonstrated for the first time about three years ago [23]. Introduction of the telomerase catalytic protein component (hTERT) into normal human cells without detectable telomerase results in restoration of telomerase activity and telomere maintenance or elongation [23-26]. Normal human cells stably expressing transfected telomerase are functionally immortal and have divided hundreds of times, providing direct evidence that telomere shortening controls cellular aging. The cells with introduced telomerase maintain mostly a normal chromosome complement and continue to grow in a normal manner. These observations provide direct evidence for the hypothesis that telomere length determines the proliferative capacity of human cells.

1.5. Replicative aging and cancer

Cellular senescence may have evolved, in part, to protect long-lived organisms, such as humans, against the early development of cancer. Cancer cells must acquire many mutations before they become malignant, and replicative aging inhibits this progression by halting cell division before more than a few mutations have accumulated. The telomere-telomerase hypothesis of aging and cancer is strengthened by the finding that most human tumors have upregulated or reactivated telomerase activity [27-54]. In contrast to normal cells, tumor cells show no net loss of average telomere length with cell division (Figure 1), suggesting that telomere maintenance may be required for cells to escape from replicative senescence and proliferate indefinitely. Most, but not necessarily all, malignant tumors [27, 29] may need telomerase to sustain their growth. Immortalization may occur through a mutation of a gene (or genes) in the telomerase repression pathway. Thus, upregulation or reactivation of telomerase activity may be a rate-limiting step required for the continuing proliferation of advanced cancers. There is experimental evidence from hundreds of independent laboratories that telomerase activity is present in approximately 90 percent of all human tumors but not

in tissues adjacent to the tumors [27-54]. Thus, clinical telomerase research is currently focused on the development of methods for the accurate diagnosis of cancer and on novel anti-telomerase cancer therapeutics.

1.6. Can telomerase be used as a product to extend cell lifespan?

The ability to immortalize human cells and retain normal behavior holds promise in several areas of biopharmaceutical research including drug development, screening and toxicology testing [55]. The development of better cellular models of human disease and production of human products are among the immediate applications of this new advance. This technology has the potential to produce unlimited quantities of normal human cells of virtually any tissue type and may have its most immediate translational applications in the area of transplantation medicine. In the future it may be possible to take a persons own cells, manipulate and rejuvenate them without using up their lifespan and then give them back to the patient. For example, genetic engineering of telomerase-immortalized cells could lead to the development of cell-based therapies for certain genetic disorders such as muscular dystrophy.

2. Recent misconceptions in the cell immortalization and telomerase field

There have been a number of papers published during the past two years that are accurate in regards to the observations reported but that have been misinterpreted by many scientists and science journalists and have thus led to some confusion in the telomerase and cell immortalization fields. The subjects listed in Table 1 will be discussed individually.

Table 1.

• Is telomerase insufficient to immortalize human keratinocytes and breast epithelial cells?
• Is there a difference in the frequency of immortalization between rodent and human cells?
• Is immortalization by telomerase risky?
• Can human cells be transformed by alterations in three cellular pathways?

2.1. Is telomerase insufficient to immortalize human keratinocytes and breast epithelial cells?

Replicative aging is one of the basic mechanisms that long-lived organisms have evolved for limiting the number of oncogenic mutations that accumulate before growth arrest intervenes. Telomere shortening has been shown to be one mechanism capable of counting cell divisions. The apparent failure of telomerase to immortalize a variety of epithelial cell types has led many authors to propose that the p16/RB pathway

may represent a telomere-independent mechanism for counting cell divisions during replicative aging [56-61]. We have re-examined the evidence for these claims and the results demonstrate that the p16-mediated growth arrest observed with at least two different epithelial cell types (skin keratinocytes and HMECs) is a consequence of the particular tissue culture conditions used, and can be prevented by growing the cells on appropriate feeder layers [62]. Keratinocytes immortalized with telomerase on feeder layers can still exhibit a p16-mediated growth arrest after being transferred to plastic culture dishes. Rare cells that eventually escape this checkpoint on plastic and resume growth no longer express p16, consistent with the previous demonstrations that inactivation of the INK4a locus is required for extended growth of these cells in chemically defined media on plastic substrates. However, since these cells are already immortal when grown on feeder layers, it is inappropriate to describe this change as required for immortalization. Instead, p16 inactivation should be characterized as an event necessary for proliferation in an inadequate culture environment. We thus conclude that the published evidence does not support the presence of telomere-independent mechanisms of replicative aging.

It is unambiguous that $p16^{Ink4A}$ is an important tumor-suppressor involved in cell cycle regulation. The demonstration that the p16-mediated growth arrest found in some cultured cells represents a response to inadequate culture conditions rather than a telomere-independent mechanism of replicative aging focuses attention on its role in mediating stress theoretically induced by a variety of factors, rather than in counting cell divisions [63, 64]. This will permit more effective analyses of telomeres in replicative aging, their role in tumor prevention and the possibilities of manipulating telomere biology for the treatment of cancer.

The cell cycle is regulated by positive growth factors that induce progression through the cell cycle, as well as negative regulators that can block this progression once a threshold level of inhibition is reached. As is illustrated in Figure 2 and indicated by the dotted line, there is a threshold for growth arrest by negative regulators. All tissue culture conditions probably induce a certain level of damage or stress responses to this non-physiological environment. If the level of stress plateaus below the threshold value, then cells can proliferate until additional checkpoint activities, induced by telomere shortening, intervene and cause the growth arrest of cellular senescence. However, if the culture conditions (e.g. plastic *versus* feeder layers) cause the stress response to progressively increase, then growth arrest can occur before the effects of replicative aging can be seen. When the cells are maintained in a culture environment that does not exceed the threshold for growth arrest until telomeres become too short, then introduction of hTERT can maintain the cells below this growth arrest threshold resulting in cell immortalization.

2.2. Is there a difference in the frequency of immortalization between rodent and human cells?

The confusion between replicative aging and inadequate culture conditions illustrated above for human epithelial cells is similar to what is found for mouse embryo fibroblasts

[65, 66]. The growth arrest of mouse fibroblasts that occurs after only a few divisions in cell culture is telomere-independent, and we believe it has been inappropriately referred to as replicative or cellular senescence. Since this growth arrest occurs in rodent cells when there are still very long telomeres and at the normal time in cells from mice lacking the RNA component of telomerase [67] whose telomeres are shorter than those in wild-type mice, we believe this phenomenon should not be compared to telomere-based replicative senescence that occurs in human cells grown under the appropriate conditions. There is no experimental evidence that the replicative growth arrest experienced by primary rodent fibroblasts in culture depends upon a biologic clock that measures generation number. The observation that the transcriptional activity of p53 is induced 10- to 40- fold within the first three passages after mouse embryo fibroblasts are put in culture [68] suggests that the culture environment is producing DNA damage in these cells, as does the rapid growth arrest that occurs in culture using cells from mice with deficiencies in many DNA-repair factors [69-73].

Human fibroblasts have a more extended proliferative potential than their mouse counterparts in culture, yet essentially never become adapted to continuous growth as immortal cell lines ("establishment"). Human cells are more resistant than rodent cells to oncogene-mediated transformation and are more chromosomally stable. Despite these obvious differences, many reports have linked conclusions drawn from studies in these different cell systems, equating senescence in mouse and human cells. Sherr and DePinho [65] recently reviewed the idea that a growth failure of cultured cells results from two sources of signals, either of which can induce the expression of a common set of inhibitors of the cell division cycle. One source that may trigger a cellular growth arrest is extrinsic and stems from inadequate conditions cells experience when they are explanted into culture. The second class is intrinsic and depends upon cell cycle cellular machinery that monitors the integrity of telomeres. Mouse cells are more sensitive to a variety of stresses of the culture environment because these shorter-lived animals have not evolved as effective damage protection and repair machinery as longer-lived humans [74]. It is likely that culture conditions that are adequate for human fibroblasts do not support the long-term proliferation of mouse fibroblasts. However, mouse cells can overcome this early growth arrest by inactivating the ARF pathway [65, 75]. We believe this is one of the reasons that murine cells "immortalize" with a much higher frequency than comparable human cells (Table 2).

Table 2. Of mice and men

• Normal mouse cells spontaneously immortalize with high frequency
• Normal human cells do not spontaneously immortalize
• Laboratory mice are 350 times smaller yet get cancer much more frequently, per animal per year, than humans
• The shortest mouse telomere is longer than the longest human telomere
• Telomerase is repressed less effectively in mice

Finally, it is entirely possible that telomere-based replicative senescence does not exist in the mouse and only evolved in larger and longer-lived organisms as an additional restraint against tumor formation [66].

2.3. Is immortalization by telomerase risky?

Introduction of telomerase into normal cells has been shown to result in maintenance of telomeres and extension of lifespan in several types of fibroblasts, epithelial and endothelial cells [23-26, 76, 77]. Since telomerase is expressed in almost all cancer cells, it is legitimate to ask if introduction of hTERT into normal cells results in an increased risk of cancer progression. As illustrated in Table 3, it has been shown that the properties of hTERT-expressing cells are essentially identical to matched normal cells except in the ability of the cells to divide indefinitely [78, 79].

Table 3. Properties of normal, cancer and hTERT-expressing fibroblasts in culture

Characteristics	Normal	Cancer	hTERT
Contact inhibition of growth	present	absent	present
Growth factor requirements	high	low	high
Anchorage-dependence	present	absent	present
Cell. cycle checkpoints	intact	absent	intact
Karyotypic profile	normal	abnormal	normal
Proliferative life span	finite	indefinite	indefinite

It has been shown that hTERT expressing cells have similar growth rates to normal cells; that p53, p21, p16 and pRB protein levels are unchanged; DNA damage checkpoints are intact (p53 is induced after UV-B and gamma irradiation); oncogene-induced checkpoints are intact (e.g. ras induced "premature senescence" still occurs); and "telomerized" cells do not make tumors in nude mice. Thus, telomerase by itself does not cause the acquisition of any abnormal growth characteristics, but only provides the cells with unlimited divisions. Cells almost certainly would have to accumulate many additional alterations to become cancerous.

In this regard, it was recently reported that human mammary epithelial cells (HMEC) established in cell culture and expressing hTERT upregulated c-myc and expressed their endogenous hTERT when the hTERT expression vector was removed after 135 population doublings [80]. While the cells were still karyotypically normal and nontumorigenic, this was interpreted to indicate that the cells were one additional step closer to cancer (since c-myc is upregulated in most cancers) and that expressing telomerase in cells could potentially be risky. However, it is important to note that because the HMECs were grown on a plastic substrate in culture, they had almost certainly inactivated p16 [61, 62]. Normally p16 would sense cellular stresses and DNA damage and prevent abnormal cells from progressing through the cell cycle. Given that the culture conditions were inadequate, there would be a strong selection pressure for

cells that had activated growth-promoting factors such as c-myc that might help cells proliferate better. The absence of p16 is likely to have contributed to the survival of cells with elevated c-myc. Thus, the most likely explanation for the increased c-myc expression in the HMECs in this study was growing them under inadequate culture conditions that produced multiple abnormalities. In similar experiments conducted in our laboratory, hTERT was introduced into normal fibroblasts for only a short period of time, and after hTERT was excised and there was no activation of the endogenous telomerase [81]. The cells had elongated their telomeres and after removal of hTERT the telomeres began to shorten and eventually the cells stopped proliferating and underwent normal senescence (without any increase in c-myc). Our cells had normal p16, and were cultured under conditions that appear excellent for fibroblasts, so this could explain the difference between our study and the ones conducted on HMECs. Thus, while we agree it is entirely appropriate to be cautious, we also believe that putting hTERT into cells that can be well characterized would not increase the risk of cancer very much and may be worth the risk in certain cases.

2.4. Can human cells be transformed by alterations in three cellular pathways?

The research of many scientists over the previous decades has clearly documented that there are several parallel paths to tumorigenesis [82, 83]. While the exact sequence of alterations that must occur within the putative cancer cell are not fixed, there are general paths leading to tumorigenesis (Table 4) that almost all developing cancer cells must follow. Losing tumor suppressor functions such as those of p53 and p16/pRB, activation of ras signaling pathways, producing insulin-like growth survival/anti-apoptosis factors, inducing angiogenesis by producing VEGF, losing the ability to undergo normal differentiation, upregulating or turning on telomerase to maintain the telomeres, and inactivating E-cadherin to produce cell invasion through the stromal compartment are general pathways that are often affected within the putative cancer cell [82]. However, there are also extrinsic cellular factors that occur that provide a favorable environment for the growth of the cancer cell. In addition to interactions between cancer cells, the concept of a heterotypic tumor environment must incorporate the idea that inflammary cells producing proteases (Hanahan, personal communication), endothelial cells migrating towards the tumor bed, and stromal fibroblasts participating in cell-cell interactions within the tumor environment are central to cancer progression.

Table 4. Paths to tumorigenesis

• Self sufficiency in growth signals
• Insensititivy to antigrowth signals
• Loss of differentiation capacity
• Evading apoptosis
• Sustained angiogenesis
• Limitless replicative potential

Many previous attempts to convert normal human cells to tumorigenic cells have failed using protocols that efficiently convert murine cells. In an attempt to begin defining specific genetic alterations to convert a normal human cell to a tumor cell, it was reported that the ectopic expression of an hTERT cDNA in combination with two oncogenes (SV40 large T-antigen and an oncogenic allele of H-*ras*), resulted in direct tumor conversion of normal fibroblasts and epithelial cells [84]. We had conducted similar experiments using the same normal fibroblasts ectopically expressing an hTERT cDNA, HPV 16 E6/E7, plus oncogenic H-*ras* and our cells did not grow in soft agar or induce tumor formation [79]. HPV16 E6/E7, similarly to SV40 large T-antigen, abrogates p53 and p16/Rb pathways so it was not initially obvious why there was this very different result. Clearly HPV and SV40 oncoproteins could "take care of" different cellular functions. However, as it turns out, the explanation may be very simple. The original report apparently was using a vector that was expressing not only SV40 large T-antigen but also small t-antigen. Small t-antigen inhibits protein phosphatase 2A activity [85] and its presence was required for the effect (Hahn and Weinberg, personal communication). In addition it appears that extraordinary high (10-fold higher than in tumor cells) levels of ras are required. The bottom line is that the steps leading to tumorigenesis are quite complex and not yet fully understood. The key is to understand the specific genes altered in the multiple cellular pathways as outlined in Table 4.

To begin to address the question of how many "hits" does it take to transform a normal cell to a tumor phenotype, there was an interesting animal study in which bovine adrenal cells were "telomerized" with hTERT and also transfected with the viral oncoprotein SV40 large T-antigen. T-antigen takes over cell growth control (by inactivating at a minimum both the p53 and p16/Rb pathways) and hTERT immortalized the bovine cells [76]. This study showed that these hTERT, T-antigen expressing bovine adrenal cells could be placed in the kidneys of immunosuppressed scid mice. The scid mice were also adrenalectomized (so that they did not produce glucocorticoids). Such mice die within 25-days after adrenalectomy as glucocortocoids are essential for life. However, the introduced bovine cells intermingled with stromal cells of the scid mice, differentiated, and produced bovine cortisol which rescued the animals from dying. This indicates that hTERT and T-antigen expressed in cells and positioned in the correct environment are not tumor cells, but behave normally and differentiate and produce differentiated functions. Although great caution will clearly be needed if oncogenes such as T-antigen are combined with telomerase, these results emphasize how difficult it may be to make even immortal cells behave malignantly.

3. Summary and future directions

In this introductory chapter, we have reviewed the evidence that escape from senescence and crisis are important events in the life history of human cancer cells, and that the presence of telomerase activity *per se* can reflect an immortal state, but does not directly imply an oncogenically transformed one [82]. In addition, we have shown that several misconceptions about cellular senescence have resulted from signals that can occur