The use of hTERT-immortalized cells in tissue engineering

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Abstract

The use of human telomerase reverse transcriptase (hTERT)-immortalized cells in tissue engineering protocols is a potentially important application of telomere biology. Several human cell types have been created that over-express the hTERT gene with enhanced telomerase activity, extended life span and maintained or even improved functional activities. Furthermore, some studies have employed the telomerized cells in tissue engineering protocols with very good results. However, high telomerase activity allows extensive cell proliferation that may be associated with genomic instability and risk for cell transformation. Thus, safety issues should be studied carefully before using the telomerized tissues in the clinic. Alternatively, the development of conditional or intermittent telomerase activation protocols is needed.

Abbreviations: mTERT/hTERT – mouse/human telomerase reverse transcriptase; mTR – mouse telomerase RNA; K5 – keratin 5; SMC – smooth muscle cells; PD – population doublings; hESC – human embryonic stem cells; hASC – human adult stem cells; hMSC – human mesenchymal stem cells; SV40 – simian virus 40; HPV16 – human papillomavirus type 16; HSC – hematopoietic stem cells; PGA – polyglycolic acid; HA/TCP – hydroxyapatite/tricalcium phosphate.

Introduction

The emerging field of regenerative medicine holds the promise of treating a variety of degenerative and age-related diseases where no specific or effective treatment is currently available by transplanting biologically competent mature cells and organs. One of the principal sources of tissues and organs that will be employed in regenerative medicine protocols are those derived from tissue engineering. Tissue engineering implies the *ex vivo* seeding of organ-specific cells or stem cells on

bio-degradable scaffolds to enhance the formation of a particular tissue or provide 3-dimensional structures for forming a complete organ. The list of tissues and organs that can be generated through tissue engineering is large and range from generating bone grafts to be used in repairing bone defects to *ex vivo* engineering of heart valves, arteries, kidney or urinary bladder (Stock and Vacanti 2001). Furthermore, the importance of tissue engineering is increasingly recognized due to the escalating need for tissues and organs for replacement therapy since degenerative and age-

related diseases due to organ and tissue failure are currently major health care problems. Also, the possible use of cadaveric organs is limited due to the lack of sufficient number of organ donors, limitations attributable to immunological rejection and mismatch of physical dimensions.

A prerequisite for successful results of the tissue engineering approach is the ability to grow the cells in sufficient number and at the same time maintain their biological properties. Recently, several investigators have tried to introduce the human telomerase reverse transcriptase (hTERT) gene to various human cell types with the aim of obtaining large number of biologically competent cells. The present review will describe these studies and discuss them in the context of the potential use of hTERT-immortalized cells for therapy. Basic knowledge pertaining to telomeres and telomerase is covered by the other reviews published in this special issue of Cytotechnology.

Limits for proliferation of somatic cells in vitro

Normal human cells can only divide a limited number of times, after which point they enter an irreversible state of growth arrest. This phenomenon is termed replicative senescence. The maximal number of population doublings (PD) a cell can undergo is termed the Hayflick limit, and it varies depending on the cell type, donor age and culture conditions (Shay and Wright 2000a; Campisi et al. 2001; Rattan 2003). Under conventional *in vitro* culture conditions that include fetal bovine serum, human osteoblasts can grow *in vitro* up to 24–40 PD (Kassem et al. 1997). This number is similar to what has been reported in other cell types (Shay and Wright 2000a; Campisi et al. 2001). Replicative senescence of cultured cells can be caused by several factors, but the progressive telomeres shortening resulting from the lack of telomerase activity in somatic cells is emerging as a fundamental mechanism (Harley 1991; Wright and Shay 2002).

Stem cells vs. somatic cells: the telomerase connection

Stem cells are defined as cells with both self-renewal capacity and an ability to differentiate into various cell types (Watt and Hogan 2000). These biological characteristics make stem cells attractive

cell models in tissue engineering protocols (Bianco and Robey 2001). Two types of human stem cells are currently available for use in therapy. The first are human embryonic stem cells (hESC) derived from the inner cell mass of the early embryo blastocyst (Odorico et al. 2001) or primitive germ cells of around 2 months old fetal tissues (Shamblott et al. 1998). The second stem cells type is human adult stem cells (hASC) (or better terminology: tissue-specific stem cells) that are derived through specific isolation procedures from nearly every organ in the body (e.g. bone marrow, liver, pancreas, adipose tissue, dental pulp, etc). Important Differences exist between hESC and hASC. Differentiation capacity of hESC is much wider than hASC. Also, hESC exhibits enormous self-renewal capacity *in vitro* due to the presence of telomerase activity (Odorico et al. 2001). On the other hand, hASC exhibit with continuous *in vitro* cultivation a loss of telomerase activity, a gradual attrition of the telomeres and a limited life span, as well as a progressive decline in biological functions (Rao and Mattson 2001). One type of hASC that has been extensively studied by our group is the human mesenchymal stem cells (hMSC). We have found that hMSC obtained from young donors can grow up to 24–40 PD *in vitro* and that the proliferative potential of hMSC obtained from older donors is much lower (Stenderup et al. 2003). Also, the biological activity of the cells as determined by the ability to produce bone specific proteins is impaired during long-term culture (Kassem et al. 1997; Kveiborg et al. 2001; Stenderup et al. 2003). We found that hMSC do not have telomerase activity due to absence of hTERT expression (Simonsen et al. 2002). This has been supported by other investigators (Zimmermann et al. 2003). Other hASC exhibit a similar senescent phenotype during prolonged growth. For example hematopoietic stem cells (HSC) exhibit progressive telomere shortening and defective transplantability during successive rounds of transplantation (Allsopp and Weissman 2002). It is not clear whether the *in vitro* culture conditions of hASC are responsible for silencing of hTERT gene expression or hASC exhibit low levels of telomerase activity that are not detectable by the current methodologies. It is conceivable that hASC may have conditionally regulated telomerase activity that is normally low or absent *in vivo* since it is insufficient to maintain *in vivo* telomere

length and possibly the functional capacity for tissue regeneration with aging (Campisi et al. 2001).

Extension of life span and 'rejuvenation' of cells by hTERT expression

Initial studies introducing hTERT into somatic cells were aimed at testing the 'telomere hypothesis' of replicative senescence. Bodnar et al. (1998) have demonstrated that transfecting two cell types, retinal epithelial cells and foreskin fibroblasts, with hTERT resulted in elongating their telomere lengths as well as extending their life span compared to the hTERT-negative control cells that experienced replicative senescence with continuous *in vitro* growth (Bodnar et al. 1998). Interestingly, hTERT alone was sufficient to bypass the senescence-associated growth arrest in a number of cell types, including fibroblasts, osteoblasts, endothelial cells, keratinocytes, and several types of epithelial cells (Wada et al. 2003). However, in some cases life span extension was not possible and in others ectopic expression of other genetic elements (e.g. SV40 large T antigen or HPV16 E6/E7) was necessary for cell immortalization (Wada et al. 2003). The explanation of these differences is not clear at the present time but may be related to integration site or dose of the transgene or the cell type utilized.

From a tissue engineering point of view, maintenance of the functional activity of the cells is more relevant than cell proliferation alone. Thus, several investigators have examined the effect of hTERT overexpression on the biological functions of the cells. The overall results have been very consistent and impressive. hTERT overexpression resulted in maintenance or even enhancement of the biological activity of various cell types. hTERT overexpression in endothelial cells led to better mitogenic activity and an improved neo-vascularization after *in vivo* implantation compared to control telomerase-negative cells (Yang et al. 2001; Murasawa et al. 2002). Also, telomerized human fetal hepatocytes exhibited an extended life span and maintenance of their liver-specific characteristics *in vitro* and *in vivo* (Wege et al. 2003). Challenging the understanding that senescence is an irreversible phenotype, the possible cellular 'rejuvenation' by hTERT overexpression was also

examined in senescent cells. hTERT was introduced into senescent fibroblasts and their gene expression profile and behavior *in vivo* were examined in a reconstituted human skin model. Telomerized senescent fibroblasts behaved similar to young fibroblasts (Funk et al. 2000). Also, overexpression of hTERT in senescent osteoblasts improved their functional capacity for bone formation as determined by implantation in a model for *in vivo* bone formation (Yudoh and Nishioka 2004). The positive effects of telomerase activity on the biological functions of the cells was not restricted to differentiated cells but also has been also demonstrated in hASC. Over-expression of hTERT in hMSC led to an extended life span with better bone forming capacity in an *in vivo* model for bone formation (Figure 1) (Shi et al. 2002; Simonsen et al. 2002) and also enhanced gene expression of osteoblast-specific genes as determined by gene expression profiling (Gronthos et al. 2003). Finally, impaired differentiation capacity of MSC from telomerase deficient mice (mTR^{-/-} mice) has been demonstrated suggesting a role for telomerase activity in maintenance of MSC biological integrity (Liu et al. 2004).

The above findings in cell cultures were furthermore supported by developing transgenic mice models where TERT is expressed under the influence of tissue-specific promoters. hTERT expression driven by mouse α MHC promoter to obtain cardiac specific expression, resulted in increase cardiomyocyte density and protection against apoptosis after ischemic injury and consequently reduction of the area of infarction (Oh et al. 2001). Gonzalez-Suarez et al. (2001) developed a transgenic mouse where mouse TERT (mTERT) expression was targeted to basal keratinocytes and various stratified epithelia using bovine keratin 5 (K5) promoter (K5-mTERT). This resulted in histologically normal stratified epithelia with improved wound healing rate compared to wild-type littermates. Finally, mice with generalized ectopic expression of mTERT under the β -actin promoter, were resistant to ischemic injury of the brain after occlusion of the middle cerebral artery (Kang et al. 2004). On the other hand, mTERT overexpressed in hematopoietic stem cells (HSC) under the major histocompatibility class I gene *H-2K^b* promoter, did not result in improved HSC transplantation capacity (Allsopp et al. 2003b). This is an inconsistent finding since HSC

Figure 1. (a) Growth curves in long-term culture of human mesenchymal stem cells (hMSC) and hMSC over-expressing human telomerase reverse transcriptase gene (hMSC-TERT). (b) The cells were implanted subcutaneously in immunodeficient mice together with hydroxyapatite/tricalcium phosphate (ha). The cells formed ectopic bone and bone marrow organ. (b – bone, m – marrow, a – adipocytes), (c) The amount of bone formed was quantitated using histomorphometric methods. BV/TV: bone volume per total volume, PD – population doubling (from Simonsen et al. 2002)

from telomerase deficient mice revealed severely reduced transplantation capacity compared with wild type controls (Allsopp et al. 2003a).

All these data suggest that telomerase activity plays a wider role in maintaining cellular and biological functions than just merely telomere maintenance. These functions are highly relevant to the potential use of 'telomerized tissues' in tissue engineering and include anti-apoptotic effects, effects on differentiated cellular functions, genomic stability and DNA repair. These novel functions for telomerase have been termed 'extracurricular activities' (Chang and DePinho 2002).

Validation of the concept: using telomerized cells in tissue engineering

Based on the above mentioned evidence, some investigators have tested the possibility of using telomerized cells in tissue engineering protocols (Shay and Wright 2000b). Table 1 shows a variety of cell types where telomerized cells have been created with the potential for use in tissue engineering. Some of the outstanding examples are

presented here. McKee et al. (2003) overexpressed hTERT in human smooth muscle cells (SMC) that were seeded onto tubular scaffolds of degradable polyglycolic acid (PGA) mesh and were cultured under pulsatile pressure in bioreactors (McKee et al. 2003). After seven weeks, the formed blood vessels were seeded with endothelial cells. The blood vessels formed using hTERT-SMC were much better compared with those derived from control cells with greater wall thickness, higher cell density, reduced cellular apoptosis and higher rupture strengths (McKee et al. 2003) (Figure 2). Similarly, hTERT-expressing hMSC seeded in hydroxyapatite/tricalcium phosphate (HA/TCP) scaffolds and implanted subcutaneously in immune-deficient mice formed more bone of normal quality compared with implants of normal cells (Simonsen et al. 2002) (Figure 1). Thomas et al. (2000) implanted bovine adreno-cortical cells overexpressing hTERT and SV40 large T antigen genes in a 3 mm polycarbonate cylinder beneath the renal capsule of adrenalectomized immune-deficient mice. Control animals without transplantation died of adrenal failure whereas animals that received gene modified adreno-cortical cells sur-

Table 1. Examples illustrating the versatility of telomerized cells and their potential use in tissue engineering and therapy.

Cell	Extended lifespan	Potential clinical application	References
Bovine adrenocortical cells	+ SV40T*	Transplant correction of hormone deficiency states	Thomas et al. (2000)
Human dermal fibroblasts	Yes	Skin wound healing	Funk et al. (2000)
Human fetal hepatocytes	Yes	Treatment of liver diseases	Wege et al. (2003)
Human dermal microvascular endothelial cells	Yes	Vascular regeneration	Yang et al. (2001)
Human smooth muscle cells	Yes	Engineered blood vessels	McKee et al. (2003)
Human Chondrocytes	+ HPV16 E6/E7*	Cartilage repair and regeneration	Martin et al. (2002)
Human retinal pigment Epithelial cells	Yes	Repair retinal damage and degeneration	Rambhatla et al. (2002)
Human satellite myoblasts	Yes	Repair muscle degeneration	Di Donna et al. (2003)
Human mesenchymal stem cells	Yes	Repair bone defects	Simonsen et al. (2002)
Human osteoblasts	Yes	Repair bone defects	Yudoh and Nishioka (2004)

*Refers to the need of additional genetic elements for cell immortalization.

Figure 2. Telomerase enables robust vascular tissue engineering. Hematoxylin-eosin staining of smooth-muscle cells (SMCs) untransduced (a) or stably transduced with gene hTERT (b). Scale bar = 0.5 mm. Table shows the physical characteristics of engineered blood vessels. ^aMean $\pm$ SEM of four samples. ^b $p < 0.01$; ^c $p < 0.0005$ (from McKee et al. 2003).

vived and produced bovine cortisol that replaced the mouse glucocorticoids (corticosterone). In addition, the gene-modified cells did not form tumors (Thomas et al. 2000). This study demonstrates the possibility of using hTERT-overexpressing cells for treatment of hormone deficient states.

Genomic stability of telomerized cells

In addition to the efficacy of the transplanted tissues and organs, safety issues are important and need to be addressed before introducing telomerized tissues in clinical trials. Earlier extensive studies of hTERT overexpression in human diploid cells (fibroblasts and retinal pigment epithelial cells) revealed maintenance of the genetic integrity and absence of the transformed phenotype (Jiang et al. 1999; Morales et al. 1999). We have tested the

effects of long-term overexpression of hTERT in hMSC on the cellular and genomic stability of the cells, during *in vitro* culture period over 3 years. At PD 256, the cells showed loss of contact inhibition, anchorage independence and formed tumors in 10/10 mice (Serakinci et al. 2004). Interestingly, chromosomal analysis of the cells showed a consistently normal [46, XY] karyotype. However, DNA analysis revealed the acquisition of genetic and epigenetic changes, which included a deletion of the *INK4a/ARF* locus, activating mutation in *KRAS*, and hypermethylation of the cell cycle regulating gene *DBCCR1*. Our results corroborate previous findings in TERT transgenic mice of increased tumor susceptibility. For example in K5-mTERT transgenic mice an increased sensitivity to the mitogenic effects of phorbol esters as well as increased incidence of preneoplastic and neoplastic lesions have been reported (Gonzalez-Suarez et al. 2001). Also, in mice over-expressing mTERT under the β -actin promoter, an increased incidence of mammary carcinoma was observed during aging of the animals (Artandi et al. 2002). Finally, the genetic changes in telomerized hMSC does not seem to be cell type specific phenomenon since similar but less pronounced changes were recently reported in other cells types (Milyavsky et al. 2003; Noble et al. 2004).

Concluding remarks and future perspectives

The biological rationale for using telomerized cells in tissue engineering is well supported by ample of

in vitro and *in vivo* evidence. However, the recent findings of the potential induction of genetic changes and the transformed phenotype in the hTERT overexpressing hMSC, suggest that safety issues should be a major concern before introducing the telomerized cells in clinical trials. Alternative methods for 'telomerization' of the cells should be explored. For example, inducible hTERT expression using chemical agents (Huang et al. 2003) or the use of conditional promoters driving hTERT expression (Rubio et al. 2004) are two viable possibilities. Also, understanding hTERT and telomerase biology as well as the mechanisms and the role of numerous co-factors involved in regulating their activities are important prerequisites for devising strategies for either induction or silencing telomerase activity. It is also important to note that phenotypic drift towards tumorigenicity is not an inevitable outcome of hTERT-immortalized cells. We have observed in our laboratory some hMSC-TERT strains that have been growing for more than 3 years in a high density cultures that allow a more controlled growth environment with greater intercellular contact and communication and they still exhibit normal growth and no evidence of transformation (J.S. Burns and M. Kassem, unpublished data). Thus, future studies are needed to elicit the optimal culture conditions that maintain normal growth and differentiation of the telomerized cells. In this regard, isolation of adult stem cells with naturally regulated telomerase activity may provide a safer cell type for tissue engineering applications compared to genetically modified cells (Jiang et al. 2002). However, the possibility of obtaining a large number of these cells with maintained genomic stability during long-term culture remains to be determined.

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