Stem Cell Transplantation

Enhanced long-term survival, but no increase in replicative capacity, following retroviral transduction of human cord blood CD34+ cells with human telomerase reverse transciptase

Overexpression of telomerase reverse transcriptase (hTERT) can immortalize some primary human mesenchymal cells.[1,2] We investigated whether retrovirally–mediated expression of hTERT in CD34+ umbilical cord blood (UCB) cells can extend the replicative lifespan of human hematopoietic progenitor cells. Overexpression of hTERT did not immortalize these cells but did lead to enhanced survival of mature hematopoietic cells.

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Telomerase is a complex ribonucleoprotein enzyme that is responsible for maintaining telomere length in germ cells and hematopoietic stem cells. The catalytic subunit of telomerase, hTERT, plays a fundamental role in telomere preservation and cell proliferation.[3] In this study CD34+ UCB cells[4] were transduced with one of a series of Moloney-murine leukemia virus-based vectors that expresses hTERT.[5,6] Control cells were transduced with an analogous vector lacking the hTERT gene. Typical titers for the vector preparations were 1-2×10^6 infectious units/mL. At the time of transduction, 90±10% of cells expressed CD34, of which greater than 50% were likely to have been transduced with hTERT.[4] Following retroviral transduction, cells were maintained under serum-free conditions in the presence of Flt3 ligand (FL) (100 ng/mL) and interleukin-3 (IL-3) (5 ng/mL). During the exponential growth phase cultures were demi-depopulated when approaching confluence and an equal volume of fresh media added. Once cell number had ceased to increase, cultures were maintained by replacing half of the media with fresh media weekly. Twelve different samples were used to establish the cultures and, when possible, duplicate cultures were maintained to reduce sampling bias.

While telomerase activity was observed in both control and hTERT-transduced cultures during the first two weeks of culture, after 2 weeks, cells in the hTERT-transduced cultures consistently expressed telomerase activity at higher levels and for longer periods of time than did control cultures. Telomerase activity was not detected in cells from hTERT-transduced cultures after 3 months. No significant differences in telomere length were observed between hTERT-transduced and control cultures at any time points examined within the first 8 weeks post-transduction. Overexpression of hTERT did not extend the replicative capacity of progenitor cells. The proportion of CD34+ cells declined rapidly to < 3% by 12 weeks and no differences were noted between the proportion or total number of CD34+ cells in the control and hTERT-transduced cultures (Table 1). The number of colony-forming cells (CFC) declined rapidly within the first 8-12 weeks of culture with no long-term differences observed between the two groups (Table 1). Overexpression of hTERT did not confer a survival advantage to UCB progenitor cells and did not override the tendency of hematopoietic cells to differentiate in the culture conditions examined.

Overexpression of hTERT in CD34+ UCB cells was found to prolong the survival of cultured mature hematopoietic cells. The cumulative cell number was measured for up to one year. No significant differences in the median maximum population doublings (PD) were observed between the control (8.9 PD) and hTERT-transduced (9.6 PD) cultures (Figure 1). After 30 weeks cell numbers began to decline; however, the total cell number in hTERT-transduced cultures was significantly higher than that in control cultures at progressive time points. By 50 weeks approximately five-fold more cells remained in the hTERT-transduced cultures than in the control cultures (p <0.01, n=7). Most cells had a *macrophage-like* morphology. FACScan analysis of cells demonstrated that long-term cultured cells expressed CD14, CD33, CD38 and E6 typical of mature myeloid cells (Table 1), suggesting that hTERT had prolonged the survival of mature myeloid cells in these cultures. Retroviral hTERT appeared to be expressed for only the first few months *in vitro,* at least to levels detectable by our assays. An early effect of hTERT may be to extend the lifespan of more primitive cells and thus generate the increased numbers of mature progeny observed at later time points.

While immortalization of some primary mesenchymal cells occurs following enforced expression of hTERT,[1,2] we found no evidence for hTERT-induced immortalization of hematopoietic cells under the culture conditions employed in the current study. The major finding of this study, that retroviral-mediated expression of hTERT in human UCB CD34+ cells pro-

Table 1. Summary of hematopoietic cell content over time.

	Progenitor Cells								*Mature Cells*					
Time	*Mix-CFC*[a]		*GM-CFC*[a]		*BFU-E*[a]		*CD34+*[b]		*CD14+*[b]		*CD33+38+*[b]		*E6+*[b]	
(wks.)	*Cont*	*hTERT*	*Cont*	*hTERT*	*Cont*	*hTERT*	*Cont*	*hTERT*	*Cont*	*hTERT*	*Cont*	*hTERT*	*Cont*	*hTERT*
0	ND	ND	ND	ND	ND	ND	$2.5x10^5$	$2.5x10^5$	$3.6x10^3$	$3.6x10^3$	$2.7x10^5$	$2.7x10^5$	$2.9x10^4$	$2.9x10^4$
0.4	242	300	918	714	1233	1003	$8.5x10^5$	$9.9x10^5$	$7.9x10^4$	$7.8x10^4$	$9.7x10^5$	$1.1x10^6$	$2.4x10^5$	$2.9x10^5$
1	136	52	849	670	814	865	$3.4x10^6$	$3.9x10^6$	$2.8x10^5$	$5.0x10^5$	$5.6x10^6$	$6.3x10^6$	$3.2x10^6$	$3.4x10^6$
4	13	10	676	544*	188	182	$7.2x10^6$	$7.2x10^6$	$5.3x10^6$	$5.9x10^6$	$2.3x10^7$	$2.4x10^7$	$1.4x10^7$	$1.5x10^7$
8	0.7	35	166	155	24	17	$1.1x10^7$	$1.2x10^7$	$9.1x10^7$	$3.2x10^7$	$8.3x10^7$	$8.2x10^7$	$3.5x10^7$	$3.4x10^7$
12	2.5	2.2	38	42	0.1	1.1	$1.8x10^6$	$6.1x10^6$	$5.1x10^7$	$7.3x10^7$	$1.8x10^8$	$2.0x10^8$	$8.8x10^7$	$9.3x10^7$
16	0	0	0.2	0.1	0.15	0	$1.2x10^6$	$1.5x10^6$	$1.4x10^8$	$1.8x10^8$	$1.3x10^8$	$1.6x10^8$	$1.3x10^8$	$1.7x10^8$
20	0	0	4	4	0	0	$1.5x10^6$	$1.4x10^6$	$1.7x10^7$	$4.6x10^7$	$7.6x10^7$	$1.0x10^{88}$	$2.8x10^7$	$5.2x10^7$
30	0	0	0	0	0	0	$3.6x10^5$	$1.7x10^6$	$3.2x10^6$	$2.3x10^7$	$2.0x10^7$	$4.6x10^7$	$5.3x10^6$	$1.5x10^7$
40	ND	ND	ND	ND	ND	ND	$6.4x10^4$	$1.0x10^5$	$2.2x10^6$	$4.5x10^6$	$5.9x10^6$	$1.4x10^7$	ND	ND
50	ND	ND	ND	ND	ND	ND	0.0	$1.3x10^4$	$2.4x10^3$	$1.5x10^4$	$4.0x10^5$	$8.6x10^5$	ND	ND

*Progenitor cells were assessed using flow cytometry to measure CD34+ cells and CFC assays. Mature cells were identified using flow cytometry to detect antigens expressed on differentiated myeloid cells; CD14, CD33 and CD38 for monocyte/macrophage cells and E6 (6) for erythroid cells. [a]Number of colonies per 10,000 cells, results are the mean of between 4 and 9 samples. [b]Mean total number of cells, results are the mean of between 4 and 10 samples. ND = not done. *p <0.05, n=9.*

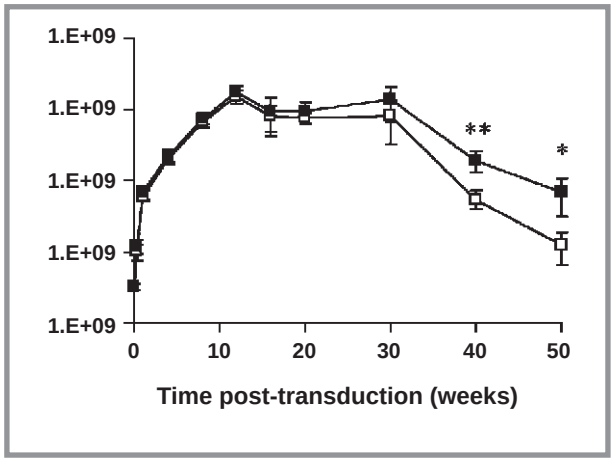

Figure 1. Long – term culture of retrovirally transduced CD34⁺ UCB cells. Cumulative cell number following transduction with control vector (open squares) or hTERT-retrovirus (closed squares) and maintenance in FL+IL3. Results are mean ± sem of between 6 and 12 samples **$p < 0.005$, *$p < 0.01$.

longs the survival of mature hematopoietic cells, was unexpected and intriguing. Limited numbers of cells were available at very late time points to perform molecular and cellular analyses and the mechanism of action for this pro-survival role of hTERT is as yet unknown. A pro-survival action of hTERT independent of telomerase enzymatic activity has recently been described in human breast cancer cells.[7] Future studies aim to elucidate the cellular and molecular mechanisms underlying this pro-survival effect of hTERT in human hematopoietic progenitor cells.

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Infectious Disorders

Clinical significance of breakthrough fungemia caused by azole-resistant *Candida tropicalis* in patients with hematologic malignancies

A 5-year retrospective analysis of fungemia in patients with hematologic malignancies revealed that four patients, who received fluconazole and itraconazole during neutropenia, developed breakthrough candidemia due to azole-resistant *Candida tropicalis* isolates. This observation suggests that causative organisms of candidemia in neutropenic patients receiving azoles should be suspected of being azole-resistant.

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Invasive candidiasis is associated with a high rate of mortality in cancer patients who develope neutropenia.[1] In these patients, *Candida tropicalis* ranks as the fourth leading cause of fungemia among *Candida* species, but blood isolates of *C. tropicalis* with reduced susceptibility to azole antifungals are very uncommon.[2-4] We present here four cases of breakthrough fungemia caused by azole-resistant *C. tropicalis* in patients with hematologic malignancies.

Between January 1, 1996, and December 31, 2000, four episodes of candidemia caused by *C. tropicalis* were identified in 701 patients with hematologic malignancies. As shown in Table 1, *in vitro* susceptibility testing according to NCCLS document M27-A revealed that all isolates had fluconazole MIC >64 μg/mL, itraconazole MIC >32 μg/mL, and voriconazole MIC >32 μg/mL, suggesting these isolates should be categorized as azole-resistant *C. tropicalis*.[3,5] Amphotericin B and micafungin were effective against these isolates with MIC of 0.0625-0.03125 μg/mL and 0.0625 μg/mL, respectively.

To evaluate the molecular epidemiology of the isolates, two primers were adopted for the randomly amplified polymorphic DNA (RAPD) analysis: R-1 (5'-ATGGATCGGC-3') and R-2 (5'-ATTGCGTCCA-3'), which had been used for the previous analysis of *Candida* species.[6] As shown in Figure 1, isolate 1 in 1996, 2 in 1997, 3 in 1997, and 4 in 1998 had distinguishing band patterns with primer R-1, suggesting the four isolates were derived from four strains of *C. tropicalis*.

Clinical data on these four patients are presented in Table 1. The underlying diseases were acute myeloid leukemia in three patients and myelodysplastic syndrome in one. All patients were profoundly neutropenic for 29-97 days because of intensive chemotherapy, and candidemia was diagnosed during the neutropenic period. All patients had central venous catheters in place and received broad-spectrum antibiotics. Surveillance cultures revealed the presence of *C. tropicalis* in oropharyngeal samples (four patients), stool samples (two), and urine samples (one),