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Granulocyte colony-stimulating factor administration upregulates telomerase activity in CD34$^+$ haematopoietic cells and may prevent telomere attrition after chemotherapy

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Abstract

Summary. Hematopoietic reconstitution could be associated with premature ageing of the transplanted cells and a high frequency of myelodysplastic syndrome and secondary leukaemia. Telomere length decreases with cell divisions and age, and at a crucial length it is associated with chromosomal instability and cell senescence. Telomerase is a reverse transcriptase enzyme that adds nucleotides to chromosomal ends. Most somatic cells lack telomerase activity yet haematopoietic stem cells retain low levels of telomerase. Some studies have found that chemotherapy and stem cell transplantation lead to the accelerated shortening of telomere length. As granulocyte colony-stimulating factor (G-CSF) is routinely used in the mobilization of stem cells for transplantation, we evaluated its effects on telomerase activity and regulation, and on telomere dynamics, in normal donors and selected lymphoma patients. Administration of G-CSF increased telomerase activity in CD34$^+$ haematopoietic cells compared with controls. In marrow-derived CD34$^+$ cells, telomerase activity increased sevenfold, compared with a 14-fold increase in peripheral-blood-mobilized CD34$^+$ cells. A parallel increase in the expression of human telomerase enzyme reverse transcriptase RNA and protein kinase C α occurred. In addition, G-CSF administration to five lymphoma patients after consecutive courses of CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy, resulted in telomere length preservation or elongation, as opposed to marked attrition in patients who did not receive growth factors. We conclude that the *in vivo* administration of G-CSF prevents or attenuates telomere attrition associated with chemotherapy administration. This attenuation may contribute to the preservation of telomere integrity inG-CSF-primed transplanted stem cells.

Over the past few decades, the widespread application of bone marrow transplantation (BMT) and peripheral stem cell transplantation (PSCT) as curative or supportive measures for patients with haematological malignancies has caused a significant increase in patients' life expectancy. However, this longer survival has revealed a high frequency of solid malignancies, myelodysplastic syndromes (MDS) and secondary leukaemia (**Witherspoon *et al*, 1989**; **Curtis *et al*, 1997**; **Engelhardt *et al*, 1998**). As clonal disorders in blood cells increase greatly with age, it has been postulated by several groups that the reconstitution of haematopoiesis after BMT or PSCT is associated with accelerated or premature ageing of the transplanted stem cells (**Engelhardt *et al*, 1998**; **Shay, 1998**; **Wynn *et al*, 1998**).

Telomeres are non-coding DNA sequences that cap the ends of chromosomes, and protect against deletions, end-to-end fusion and recombination of the proximal DNA (**Gall *et al*, 1995**). In humans, telomeric DNA consists of (TTAGGG)n repeats of 3–12kb length. As telomere length decreases with cell divisions as well as with age (**Lansdorp, 1995**), the telomere has been described as the cellular ‘mitotic clock’ and telomere length as a predictor of the remaining cellular replicative potential (**Harley *et al*, 1990**; **Allsopp *et al*, 1992**; **Lansdorp, 1995**). Telomere shortening to its critical length, the Hayflick limit (~3kb), is associated with replicative senescence (**Harley *et al*, 1990**; **Allsopp *et al*, 1992**; **Allsopp & Harley, 1995**) and chromosomal instability (**Metcalfe *et al*, 1996**). Telomere shortening during cell division can be attenuated by upregulation of the enzyme telomerase, which adds TTAGGG repeats to chromosomal ends (**Greider & Blackburn, 1989**). The rate-limiting component of the enzyme is its reverse transcriptase moiety: hTERT (human telomerase enzyme reverse transcriptase). The latter's activity is regulated at the transcriptional level by c-myc and sp1 (**Sangtaek *et al*, 1999**), and at the post-translational level through phosphorylation by protein kinase C α (PKCα) and Akt (**Kang *et al*, 1999**; **Liu, 1999**; **Yu *et al*, 2001**). Telomerase activity (TA) is absent from most somatic cells or present at very low levels in some somatic cells like stem cells (**Counter *et al*, 1992**; **Kim *et al*, 1994**). Although haematopoietic stem cells retain some TA, the telomere length of both early progenitors and mature cells decreases with age (**Engelhardt *et al*, 1997**; **Shay, 1998**), this decrease being dependent on the replicative stress imposed upon the cells (**Chang & Harley, 1995**).

Recent studies demonstrated shortening of telomeres after chemotherapy (our own unpublished observations; **Engelhardt *et al*, 1999**; **Schroder *et al*, 2001**). Several studies have reported that recipients of BMT or PSCT have shorter telomeres than their donors, this shortening being equivalent to 15–40 years of ageing (**Notaro *et al*, 1997**; **Akiyama *et al*, 1998, 2000**; **Wynn *et al*, 1998**; **Lee *et al*, 1999**). It has been suggested that telomere shortening in the transplanted stem cells could account, at least in part, for the increase in myelodysplastic syndrome (MDS) and secondary malignancies in long-term survivors of BMT and PSCT (**Shay, 1998**). In these settings, marked telomere shortening secondary to the replicative stress of bone marrow repopulation could lead to genetic instability and increased risk of mutations, leading to MDS and secondary leukaemias a few years after the transplantation. Telomere length dynamics in

haematopoietic cells depend also on TA. As such, TA in mobilized stem cells that are due for transplantation should be explored.

Granulocyte colony-stimulating factor (G-CSF) is routinely used for the mobilization of haematopoietic stem cells prior to their collection for transplantation. Engelhardt *et al* (1997) investigated telomerase activation and telomere length changes in *ex vivo* cultures of haematopoietic stem cells in response to cytokine stimulation. They found that *in vitro* exposure of $CD34^+$ cells to cytokine mixture is associated with upregulation of TA (Engelhardt *et al*, 1997). Only a few studies have addressed the effect of growth factor stimulation on telomere dynamics and telomerase in human haematopoietic stem cells.

No study to date has characterized telomerase regulation in stem cells *in vivo*, and no data has been published showing the *in vivo* effects of G-CSF administration on telomere length and TA in stem cells. In this study, we examined the effect of G-CSF administration on TA and the regulation of stem cells obtained from bone marrow or peripheral blood collected for transplantation, and its possible implications on telomere dynamics following chemotherapy.

Materials and methods

Patients. Bone marrow (BM) aspirates were obtained from five lymphoma patients with no bone marrow involvement 48 h after single administration of 5 μg/kg recombinant human (rh)G-CSF (Neupogen, Roche, Switzerland). Our preliminary experiments showed that TA increased 24 h after G-CSF administration and reached a plateau after 48 h (not shown). Peripheral blood (PB) samples were obtained on d 5 of G-CSF administration from 14 normal donors scheduled for stem cell collection. Control bone marrow aspirates were obtained from five normal volunteers. Our preliminary results showed that telomerase levels in uninvolved bone marrow from lymphoma patients are similar to levels found in normal control bone marrow (not shown). The institutional ethics committee approved the study and all patients signed an informed consent prior to sample collection.

Blood samples for telomere length determination were taken from patients with lymphoma scheduled for CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy. Five patients who received G-CSF between CHOP courses, according to their physician's decision, were compared with patients who did not receive any growth factors after the chemotherapy. The telomere length was determined prior to chemotherapy and after two cycles of treatment.

$CD34^+$ cells isolation. For $CD34^+$ cell selection, mononuclear cells were isolated from BM aspirates or leukapheresis units by Ficoll–Paque (d = 1·077 g/ml; Pharmacia, Uppsala, Sweden), rinsed and adherence-depleted overnight. After removal of adherent cells, $CD34^+$ cells were isolated using a magnetic cell-sorting program Mini-MACS (Miltenyi Biotec, Auburn, CA, USA) and the CD34 isolation kit in accordance with the manufacturer's recommendations. The purity of CD34-selected cells was determined for each isolation by FACScan (Becton Dickinson,

San Jose, CA, USA) using a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) that recognizes a separate epitope of the CD34 molecule (HPCA-2; Becton Dickinson). The yield of $CD34^+$ cells ranged from 85% to 98%.

Telomeric repeat amplification protocol (TRAP) assay. Measurement of telomerase activity (TA) was performed by the PCR-based TRAP assay, using the $TRAP_{EZE}$ telomerase detection kit (Intergene, NY, USA), according to the manufacturer's instructions and as described by **Kim *et al* (1994)**. Briefly, isolated cells were incubated with ice-cold CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate) lysis buffer for 30 min at 4°C (1 × 10^6 cells/100 μl) and subsequently were centrifuged at 13 000 r.p.m. for 30 min at 4°C. The supernatant was then collected and the protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA, USA). Protein extract (1 μg) was assayed for TRAP analysis. Each reaction was performed in 50-μl reaction mixture containing 10 x TRAP buffer, dNTP mix, TS primer, TRAP primer mix and Taq polymerase. Reaction was performed at 30°C for 30 min and was then subjected to PCR amplification for 30 cycles of 94°C, 59°C and 72°C for 30 s each, and were separated by electrophoresis on 12·5% polyacrylamide gels, in a mini-PROTEAN^{TM} gel apparatus (Bio-Rad Laboratories). The gels were stained with gel-star nucleic acid gel stain (FMC, Rockland, ME, USA). Quantifications were performed using the MULTIANALYST software for Bio-Rad's Image analysis systems (Bio-Rad Laboratories). TA was calculated according to the following formula:

where TPG is total product generated, X signifies non-heat treated samples, X_0 signifies heat-treated samples, r is the TSR8 quantification control and r_0 is 1 x CHAPS lysis buffer control.

All results were determined from at least four to six independent TRAP assays and average activity was calculated.

Lactate dehydrogenase (LDH) activity. To rule out possible protein degradation as a cause for negative telomerase activity, LDH activity was measured in all tested samples. About 50 μg protein extract was assayed in a 200-μl reaction mixture containing: Tris buffer (61 nmol/l), EDTA (6·1 nmol/l), NADH (0·037 nmol/200 ml), sodium pyruvate (7·5 nmol/l) (All reagents were from the LDH detection kit; MERCK, Sarmstadt, Germany). Samples were incubated at 37°C for 10 min. LDH activity was determined photometrically using the HITACHI 747 spectrophotometer (Roche Molecular Biochemicals, Mannheim, Germany). LDH activity was present in all samples and displayed similar activity regardless of telomerase activity.

Telomere length analysis. The telomere length analysis was performed using the flow-fluorescence in situ hybridization (flow-FISH) method. Cells were washed in phosphate-buffered saline (without Ca^{++} and Mg^{++}) containing 0·1% bovine serum albumin (BSA), and 3 × 10^5

viable cells were assayed. Following centrifugation for 15 s at 13 000 r.p.m., the supernatant was removed and cell pellets suspended in hybridization mixture containing 70% deionized formamide (Gibco, Rockville, MD, USA), 20 mmol/l Tris, pH 7·0, 1% BSA and either 0·3 μg/ml telomere-specific FITC-conjugated $(C_3TA_2)_3$ PNA probe (PerSeptive Biosystems, Framningham, MA, USA) or a similar volume of water. Samples were subjected to heat denaturation at 80°C for 10 min, followed by hybridization for 2 h at room temperature in the dark. Cells were washed twice with 1 ml of wash buffer containing 70% formamide, 10 mmol/l Tris, 0·1% BSA and 0·1% Tween 20 (Sigma), and once with phosphate-buffered saline (PBS), 0·1% BSA and 0·1% Tween 20. All washes were carried out at 16°C, the first two for 10 min at 3000 r.p.m. and the last wash for 5 min at 2000 r.p.m. Following the last wash, cells were resuspended in PBS, 0·1% BSA and 10 μg/ml RNAse A (Sigma) and propidium iodide (Sigma). Cells were transferred to Falcon 2058 tubes, incubated for 2 hours at room temperature and overnight at 4°C, and analysed on an Epics XL Flow cytometer (Beckman-Coulter, Nyon, Switzerland). Telomere length was calculated according to a calibration curve obtained with FITC-labelled beads (Quantum 824, Bangs Laboratories, IN, USA). The resulting MESF units (mean equivalent soluble fluorochrome) were converted into kbp by means of the slope for lymphocytes described previously by **Rufer *et al* (1998**).

Telomere length was determined in mononuclear cells, as it was already established that their length parallels the telomere length in $CD34^+$ stem cells. Telomere length results were expressed in relation to the normal age-related curve.

Reverse-transcription polymerase chain reaction (RT-PCR) for hTERT and PKCα. Expression of hTERT gene and PKCα was performed by a semiquantitative multiplex RT-PCR technique. Total RNA was extracted from cells using the Purescript RNA isolation kit (Gentra systems, Minneapolis, MM, USA), according to the manufacturer's instructions. RNA (1 μg) was then reverse transcribed into single-stranded DNA with Superscript^{TM}II RNase Reverse Transcriptase (Gibco BRL).

Each RT-PCR reaction was performed with hTERT- or PKCα-specific primers as well as β-actin primers as an internal control. RT PCR products were separated on 2% Nusiev gels.

hTERT mRNA was amplified by PCR using the following primers: forward primer 5′-CGG AAG AGT GTC TGG AGC AA-3′, corresponding to GeneBank positions 1785–1804, accession number AFO 18167 (**Poremba *et al*, 2000**). The reverse primer was: 5′-CTC CCA CGA CGT AGT CCA TG-3′ GeneBank position 1961–80, generating a 190-bp PCR product. Amplification was performed with 28 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s.

The β-actin primers sequences were forward primer5′-GAC CAC ACC TTC TAC AAT GAG-3′ and reverse primer 5′-GCA TAC CCG TCG TAG ATG GGG-3′.

PCR program for PKCα: 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. The forward primer was 5′-CGA GGA AGG AAA CAT GGA ACT CAG-3′, corresponding to positions 908–926,

GeneBank accession number X53479, and the reverse primer was 5′-CCT GTC GGC AAG CAT CAC CTTT-3′ (position 1101–1079)(Oshevski *et al*, 1999).

Cell cycle analysis. Frozen cells were thawed quickly in Roswell Park Memorial Institute (RPMI)-1640 medium at 37°C, washed once with RPMI-1640 medium and the cell count was performed by trypan blue exclusion. Cells (5×10^5) were stained with propidium iodide in the presence of RNAse (DNA-prep staining kit by Beckman-Coulter). The cellular DNA content was measured on an EPICS-XL flow cytometer (Beckman-Coulter). Evaluation of the DNA histogram derived from flow cytometry was made by means of the MULTICYCLE program (Phoenix Flow Systems, CA, USA). Normal human mononuclear cells were analysed concurrently with each run in order to establish the location of G_0/G_1 peak.

Results

Telomerase activity in $CD34^+$ cells at baseline and followingG-CSF administration in BM and PB samples

$CD34^+$ cells were isolated as described in the *Materials and methods* and were analysed for telomerase activity. Figure 1 depicts the TA in representative samples and Table I summarizes the values of telomerase activity. $CD34^+$ cells aspirated from baseline marrow exhibited very low TA. Forty-eight hours after G-CSF administration, telomerase activity in $CD34^+$ cells purified from BM was increased about sevenfold, and in $CD34^+$ cells collected from PB TA was markedly elevated, about 14-fold compared with baseline average levels.

Figure 1

Open in figure viewer | PowerPoint

Telomerase activity in $CD34^+$ cells. $CD34^+$ cells were isolated as described in *Materials and methods*. Telomerase activity was determined using the PCR-based TRAP assay. Representative products are shown. Lanes 1–6: BM prior to G-CSF administration; lanes 7–8: BM following G-CSF administration; lanes 10–13: PB after 5 d of G-CSF treatment. M, molecular weight marker; R8, internal TRAP standard.

Table I. Telomerase activity, hTERT and PKCα expression in bone-marrow- and peripheral-blood-derived haematopoietic cells, following G-CSF administration.

	Mean telomerase activity (TPG)	hTERT expression	PKCα expression	Percentage of cells in $S+G_2/M$ phase

	Mean telomerase activity (TPG)	hTERT expression	PKCα expression	Percentage of cells in S+G_2/M phase
Bone marrow control (no G-CSF) *n* = 5	12 ± 9	–*	–*	11–13
Bone marrow (+ G-CSF) *n* = 5	72 ± 14	+*	+*	11–13
Peripheral blood (+ G-CSF) *n* = 14	134 ± 32	+*	+*	11–13

* In all the samples tested.

Bone marrow cells were harvested and purified 48 h after G-CSF administration. Peripheral blood cells were collected from normal donors after 5 d of G-CSF administration. Telomere activity is expressed in TPG units (Total Product Generated). Expression of hTERT and PKCα transcripts was evaluated by RT-PCR. Transcripts were present in bone marrow and peripheral blood cells after G-CSF administration, and were absent in unstimulated marrow.

Transcriptional modulation of hTERT

To clarify the mechanism by which telomerase is regulated in $CD34^+$ cells as a result of G-CSF administration, we analysed *hTERT* gene expression. Multiplex semiquantitive RT-PCR reactions were performed using primers homologous to hTERT mRNA and to β-actin, which is a constitutively expressed gene and, therefore, served as control. As shown in Fig 2, there was no expression of hTERT in baseline samples. In contrast, in cells exposed to G-CSF (either from BM or from PB), hTERT mRNA was clearly expressed, in correlation with the telomerase activity in these cells.

Figure 2

Open in figure viewer | PowerPoint

hTERT expression. RT PCR reactions were performed using RNA prepared from $CD34^+$ cells isolated from various blood samples. Representative samples are shown. Lane 1: BM prior to G-CSF administration; lanes 2–3: PB after 5 d of G-CSF treatment; lanes4–5: BM following G-CSF administration. M, molecular weight marker.

RT-PCR of PKCα

PKCα transcript was not detected in $CD34^+$ cells from unstimulated bone marrow. $CD34^+$ cells from both BM and PB after administration of G-CSF expressed PKCα mRNA. As shown in Fig 3, cells that demonstrated telomerase activity and hTERT expression (after G-CSF

administration) also expressed PKCα compared with baseline $CD34^+$ cells that were not exposed to G-CSF.

Figure 3

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PKCα expression. RT-PCR reactions were performed using RNA prepared from $CD34^+$ cells isolated from various blood samples. Representative samples are shown. Lane 1: BM prior to G-CSF administration; lanes 2–3: PB after 5 d of G-CSF treatment; lanes 4–5: BM following G-CSF administration. M, molecular marker.

Cell cycle analysis

Telomerase activity is usually associated with the S phase of the cell cycle, as adding telomeric repeats to the 3′ end of the synthesized DNA strand is performed during DNA replication. Therefore, the cell cycle status of the haematopoietic stem cells was determined. No correlation was found between G-CSF treatment and cell cycle status of the haematopoietic cells, as in all samples, between 11% and 13% of the cells were in S/G_2M phase (Table I).

Telomere dynamics in mononuclear cells after CHOP chemotherapy with and without administration of G-CSF

Telomere length in the mononuclear cells of patients treated with CHOP chemotherapy decreased significantly after two cycles of treatment (Table II). In five patients who received G-CSF after each cycle of chemotherapy, the telomere length was preserved (one patient) or increased (four patients) (Table II).

Table II. Telomere length after two courses of CHOP chemotherapy, with and without G-CSF administration.

Patient	Age (years)	G-CSF administration	Telomere length before CHOP (bp)	Telomere length after CHOP (bp)	Percentage change
1	80	no	4500	3900	−14%
2	63	no	5100	4900	−4%
3	62	no	5100	3600	−30%
4	48	no	5600	4200	−25%
5	45	no	6000	5000	−17%

Patient	Age (years)	G-CSF administration	Telomere length before CHOP (bp)	Telomere length after CHOP (bp)	Percentage change
6	72	yes	3800	4800	+26%
7	57	yes	4200	5900	+40%
8	45	yes	5000	6800	+36%
9	65	yes	4700	5000	+6·4%
10	75	yes	3400	6500	+91%

Discussion

In the setting of haematopoietic stem cell transplantation, the repopulation stress imposed on the transplanted stem cells may cause shortening of their telomeres, as shown by several clinical studies (Notaro *et al*, 1997; Shay, 1998; Engelhardt *et al*, 1999; Akiyama *et al*, 2000; Schroder *et al*, 2001). This telomeric loss may be causally related to the high frequency of secondary solid and haematopoietic neoplasms observed after stem cell transplantation (Counter *et al*, 1992; Curtis *et al*, 1997). As telomerase has a major effect on telomere length, its regulation in stem cells is of prime importance. Only two studies have been published showing the induction of TA in stem cells in an *in vitro* system (Engelhardt *et al*, 1997; Kobari *et al*, 2000). There are many differences between *in vitro* and *in vivo* behaviour of cells, therefore, this study sought to evaluate the relationship between G-CSF administration and TA in patients scheduled for stem cell transplantation. As our aim was to assess TA and its regulation, as well as its immediate implications in the clinical pretransplant setting, we focused on the harvested stem cells only.

Administration of G-CSF increased the TA in both BM- and PB-derived $CD34^+$ cells. In preliminary studies, we found that TA in BM increased as early as 1 d after G-CSF administration (data not shown) and increased further on d 2. Owing to obvious reasons, we could not perform detailed time curves over several days. We measured the TA on the harvesting day because it was the first day in which a significant number of $CD34^+$ cells appeared in the peri-pheral blood. Interestingly enough, the induction of TA was significantly greater in PB $CD34^+$ cells than in their BM counterparts (see Table I). The TRAP values were highly reproducible among all our patients, both in BM and PB. Moreover, in two patients, we performed BM aspiration on the day of PB harvesting (as a result of clinical indications) and thus had the opportunity to measure TA in $CD34^+$ cells derived from both sources at the same time. In both of these patients, TA was twice as high in PB $CD34^+$ cells than in BM-derived $CD34^+$ cells. This difference in TA could be attributed to, or associated with, the different characteristics of BM as compared with mobilized PB-derived $CD34^+$ cells, including the proliferative status of the stem

cells, their different phenotypic profile, different expression of adhesion molecules and growth factor receptors, and differentiation status (Engelhardt *et al*, 1997; Link, 2000; Thornley *et al*, 2001).

Some authors have suggested that TA is upregulated during entry into S phase (Engelhardt *et al*, 1997), so the higher levels of TA activity in PB $CD34^+$ could be linked to higher cycling activity. Yet in our study, the percentage of $CD34^+$ cells in S phase did not differ significantly between BM and PB. Although Engelhard *et al* (1997) showed that TA was upregulated in S phase and repressed in G_0 cells, the results from this study clearly demonstrated upregulation of TA in G_0 cells after treatment with G-CSF. This apparent discrepancy could result from the previous studies' examination of TA regulation in cell cultures or lines, many of them cancerous or immortalized. Our study examines the*in vivo* behaviour of the cells, assuming that *in vivo* interactions are much more intricate and that other factors (stromal, microenviromental) are involved. It has been recently shown that increased activity of telomerase can persist for up to 14 d after the cells enter G_0 phase, and one group has demonstrated the lack of correlation between TA and cell cycle status (Holt *et al*, 1996, 1997). These studies showed an absence of TA in proliferating cells and activation of TA by mechanisms other than cell cycling (Holt & Shay, 1999). Therefore, this question remains to be addressed in further studies.

As TA is regulated by transcriptional and postranslational mechanisms, we investigated G-CSF's potential effects on telomerase at these different levels. In this study, administration of G-CSF increased the amount of hTERT RNA, i.e. it modulated TA on a transcriptional level. At this point, it is unclear through which pathways the *in vivo* exposure to G-CSF upregulates hTERT expression. C-myc has been shown to activate the hTERT promoter. The binding of G-CSF to its receptor activates several signal transducers and activators of transcription STAT1, STAT3 and STAT5 (de Koning *et al*, 1996; Shimoda *et al*, 1997; Ward *et al*, 1999; de Koning *et al*, 2000). STAT3 binds to a specific site in the c-myc promoter (Kiuchi *et al*, 1999), and disruption of STAT3 signalling has been shown to suppress c-myc. (Bowman *et al*, 2001). Other possible regulatory links between G-CSF and myc activation involve the tyrosine 764 of human G-CSF receptor, which activates p21Ras and causes the induction of c-myc expression in cell lines (de Koning *et al*, 1998), and another possible pathway involves the activation of Erk5, a member of the mitogen-activated protein kinase (MAPK) family by G-CSF, which has also been shown to phosphorylate c-myc. (Dong *et al*, 2001).

At the post-translational level, there was an increase in the transcription of PKCα in $CD34^+$ cells that were exposed *in vivo* to G-CSF (Table I, Fig 3). This finding is in agreement with *in vitro* studies showing that G-CSF stimulation of haematopoietic cells results in activation of PKC (Deshpande *et al*, 1997; Dong *et al*, 2001). Moreover, PKC phosphorylates tyrosine residues and thus activates telomerase. Although we measured PKC expression and not its activation, we suggest that the *in vivo* effect of G-CSF on telomerase regulation may be modulated through its expression and subsequent activation. Akt, another regulator of TA (Liu, 1999), has also been shown to be activated *in vitro* by G-CSF (Dong & Larner, 2000). Taken together, these data

suggest that G-CSF induction of TA is modulated by both transcriptional and post-translational mechanisms.

In contrast to our *in vivo* results, *in vitro* studies of *ex vivo* expansion of stem cells failed to induce TA by exposure toG-CSF alone (**Engelhardt *et al*, 1997**). As *ex vivo* expansion requires the combination of many growth factors, whereas in the clinical setting stem cell stimulation is easily achieved by G-CSF alone, we suggest that administration of G-CSF causes a cascade of events leading to multiple effects, some of them associated with telomerase activation.

Clinical studies comparing telomere length of haematopoietic stem cells in high proliferative stress situations, such as PSCT or repeated chemotherapy, have indicated an accelerated shortening of telomere length (**Notaro *et al*, 1997**; **Akiyama *et al*, 1998, 2000**; **Wynn *et al*, 1998**; **Lee *et al*, 1999**; **Schroder *et al*, 2001**). In these settings, upregulation of TA might attenuate or even prevent telomere attrition. To answer these questions, we looked at telomere dynamics following chemotherapy with and without G-CSF administration. Administration of two cycles of CHOP chemotherapy to lymphoma patients resulted in telomere shortening. These results are in line with recent reports of telomere shortening after chemotherapy(**Engelhardt *et al*, 1999**; **Schroder *et al*, 2001**).

However, in five patients who received G-CSF after each cycle of chemotherapy (as a result of clinical indications), telomere length was preserved (one patient) or increased (by 26–40% in three patients and by 90% in one patient) (see **Table II**). There was no significant age difference between the patients who received G-CSF (mean age 63 years) and those who did not (mean age 60 years). Interestingly, the former group's mean telomere length at baseline was significantly shorter (4220 bp *vs* 5260 bp). This difference of 1000 bp is compatible with 5–10 years of ageing(50–100 bp loss/year) and reflects the fact that these G-CSF-treated patients were heavily pretreated as compared with patients that did not receive G-CSF. We believe that the same limited bone marrow reserve that led their physicians to administer G-CSF is reflected in their shorter telomere lengths. Even though at baseline, these patients had shorter telomeres, after consecutive chemotherapy courses withG-CSF administration, their mean telomere length increased as compared with the patients that did not receive growth factors. We suggest that these findings may be due to upregulation of TA by G-CSF.

The possibility that telomerase upregulation by G-CSF compensates for telomere loss or preserves telomere length is in agreement with results of *ex vivo* expansion of $CD34^+$ cells derived from cord blood, where marked increase in TA was observed during expansion and no significant telomere shortening was observed (**Engelhardt *et al*, 1997**; **Kobari *et al*, 2000**). It is important to note that the observed dynamics in TA and telomere length of stem cells after chemotherapy and G-CSF administration represent only short-term effects and its long-term effects remain to be evaluated. In addition, data from other studies showing that repression of TA after entering cell quiescence can take up to 14 d deserves further investigation of the long-term dynamics of TA in already harvested stem cells. On one hand, the increased TA in the transplanted stem cells may attenuate the expected telomere loss during stem cell repopulation

and thus help in preservation of the bone marrow reserve. On the other hand, too generous use ofG-CSF may recruit more stem cells into the cycling state and thus reduce their replicative reserve by shortening their telomeres. It may well be that G-CSF exerts different and even opposite effects on the stem cell reserve in conventional chemotherapy and stem cell transplantation settings.

This study confirmed several reports that unstimulated $CD34^+$ cells express very low basal TA. *In vivo* stimulation of $CD34^+$ cells with G-CSF upregulates TA by both transcriptional and postranslational mechanisms. This upregulation is unrelated to the cell cycle status of the stem cells. TA is even more markedly elevated in PB $CD34^+$ stem cells. Administration of G-CSF attenuates the telomere loss associated with administration of chemotherapy, probably through upregulation of TA. Moreover, the recently published observations attributing an anti-apoptotic role to telomerase may add another important aspect to the TA upregulation in G-CSF-primed transplanted stem cells (**Fu *et al*, 1999**). The differences in TA between BM- and PB-derived $CD34^+$ stem cells may be related to the different engraftment properties of these two populations (**Thornley *et al*, 2001**). The long-term clinical implications of these findings, both on chemotherapy-induced stem cell reserve (**Mauch *et al*, 1988**) and the long-term effects of stem cell transplantation, should be further investigated.

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