hTERT Promotes Imatinib Resistance in Chronic Myeloid Leukemia Cells: Therapeutic Implications

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Abstract
Imatinib mesylate has shown remarkable efficacy in the treatment of patients in the chronic phase of chronic myeloid leukemia. However, despite an overall significant hematological and cytogenetic response, imatinib therapy may favor the emergence of drug-resistant clones, ultimately leading to relapse. Some imatinib resistance mechanisms had not been fully elucidated yet. In this study we used sensitive and resistant sublines from a Bcr-Abl positive cell line to investigate the putative involvement of telomerase in the promotion of imatinib resistance. We showed that sensitivity to imatinib can be partly restored in imatinib-resistant cells by targeting telomerase expression, either by the introduction of a dominant-negative form of the catalytic protein subunit of the telomerase (hTERT) or by the treatment with all-trans-retinoic acid, a clinically used drug. Furthermore, we showed that hTERT overexpression favors the development of imatinib resistance through both its antiapoptotic and telomere maintenance functions. Therefore, combining antitelomerase strategies to imatinib treatment at the beginning of the treatment should be promoted to reduce the risk of imatinib resistance development and increase the probability of eradicating the disease. Mol Cancer Ther; 10(4); 1–9. ©2011 AACR.

Introduction
Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder characterized by (9;22) chromosomal translocation, which results in the generation of p210Bcr-Abl, a chimeric protein endowed with constitutive active tyrosine kinase activity (1). This activity is essential for the induction of in vitro cellular transformation and in vivo leukemogenesis. The disease usually progresses in 3 phases: a relatively stable chronic phase, followed first by an accelerated phase and then by a blastic phase with acute leukemia characteristics, including genetic instability and acquisition of additional cytogenetic abnormalities. Imatinib mesylate, a competitive inhibitor of the ATP-binding site of the Bcr-Abl enzyme, has revolutionized the treatment of this particular type of leukemia (2). In vitro studies have shown that imatinib induces apoptosis in Bcr-Abl positive cell lines (3). However, despite an overall significant hematologic and cytogenetic response, 15% to 20% of the patients, especially in the advanced phase of the disease, develop resistance to imatinib and ultimately relapse (4–8). About half of these resistances are Bcr-Abl-dependent and are due to the reactivation of Bcr-Abl kinase activity by either gene amplification of Bcr-Abl or point mutations in the Abl kinase domain (9). The other half, requiring signaling pathways involved in cell proliferation and/or immortalization, remain to be characterized to propose additional therapies to either eradicate these Bcr-Abl positive imatinib-resistant clones or prevent the emergence of such resistances. This led us to consider telomerase as possibly involved in these resistances.

Telomerase is a ribonucleoprotein complex that is responsible for the maintenance of telomeres (10,11). The catalytic protein component of the telomerase complex, human telomerase reverse transcriptase (hTERT) plays a fundamental role in cellular immortalization. Furthermore, it has been established that telomerase is endowed with additional functions that are independent of the telomere elongating activity (12–14). Its activity is undetectable or low in normal somatic cells, while it is highly expressed in approximately 85% of common cancers, supporting the notion that telomerase is a relevant target in anticancer therapy. Telomerase activity is regulated at various molecular levels, including transcription, splicing, mRNA maturation and posttranslational modifications of hTERT. It has been reported that imatinib decreases telomerase activity in both Bcr-Abl positive and negative cells (15) and conversely that telomerase inhibition could enhance imatinib-induced apoptosis in
K562 Bcr-Abl positive cells (16). Furthermore, it has been recently hypothesized that long-term treatment with imatinib, through the sustained suppression of Bcr-Abl activity, could induce alternative telomerase-dependent signaling pathways, thus leading to the restoration of an abnormal proliferation of leukemic cells (17). However, no clear demonstration of this hypothesis has been provided yet. Whether telomerase could be involved in the biological effect of imatinib and whether it could contribute to the development of imatinib resistance are 2 unanswered questions so far.

We therefore evaluated the consequences of imatinib treatment on telomerase expression and activity using a pair of Bcr-Abl positive, sensitive and resistant cells and investigated the putative involvement of hTERT in the establishment of imatinib resistance. We showed that imatinib sensitivity can be restored in resistant cells by antitelomerase strategies and showed that overexpression of hTERT, first, protected sensitive cells from apoptosis induced by low concentration of imatinib and second, facilitated the establishment of imatinib resistance. These observations show that telomerase is an additional factor in the mechanisms of imatinib resistance and also suggest that antitelomerase strategies could prevent or at least delay the onset of such resistance.

Materials and Methods

Cell cultures

LAMA84-S, a CML imatinib-sensitive cell line derived from a patient in the blast crisis stage of CML and its imatinib-resistant subline (LAMA84-R), were both kindly provided by Prof. F. X. Mahon (Université Victor Segalen Bordeaux 2, France; ref. 18). Cells were immediately expanded, frozen on receipt and resuscitated every 4 months, using the original frozen stock. The cells were checked for the expression of the characteristic p210Bcr-Abl chimeric protein by immunoblot. LAMA84-R cells were resistant to imatinib because of the amplification of the Bcr-Abl gene (18). p210Bcr-Abl protein expression and imatinib sensitivity were regularly tested all through the study by immunoblot and apoptosis assay (see below), respectively. Otherwise, no additional authentication was done in our laboratory. Both cell lines were cultured in RPMI-1640 medium (PAA Laboratories) supplemented with 10% FBS, penicillin (50 IU/mL), streptomycin (50 μg/mL), and 1-glutamine (2 mmol/L; PAA Laboratories), and incubated at 37°C at 5% CO2 atmosphere (Binder Incubators). Every 2 days, cell density was determined using a Coulter counter and proliferation was represented in population doublings (PD) or total cell number. Cellular morphology was evaluated using May–Grunwald–Giemsa staining on cytosin preparations. Importantly, to maintain the resistant phenotype, LAMA84-R cells were continuously cultured with imatinib (1 μmol/L) even during the experiments. Indeed, these cells experienced a significant loss of viability and reduction of proliferation as early as 3 days after imatinib withdrawal from the medium as previously reported (19). The parental sensitive LAMA84-S cells were always maintained in parallel culture without imatinib.

Vector constructions and infections

Mig-R1-hTERT and Mig-R1-dominant negative (DN) form of hTERT (Mig-R1-DN-hTERT) vectors were obtained by subcloning the catalytic unit of human telomerase sequence from pBABE-puro-hTERT and pBABE-puro-DN-hTERT constructs (20) into the murine stem cell virus vector Mig-R1, containing encephalomyocarditis virus internal ribosomal entry sequence and green fluorescent protein (GFP) as a reporter gene (21), so that 59 viral long terminal repeat promoter drives its expression as previously described (22). The Mig-R1 constructs were transfected into Phoenix retroviral packaging cell line (derived from HEK293) by using Fugene HD (Roche Diagnostics), to produce (HSV-G pseudotyped) viral supernatants that were harvested 2 days posttransfections. Mig-R1 control vector Mig-R1-hTERT and Mig-R1-DN-hTERT supernatants were harvested for further LAMA84 cell infections, which were carried out in the presence of 4 μg of Proteamine-sulfate/mL (Sigma). Infected LAMA84 cells were sorted several days later for GFP fluorescence.

Drugs

Imatinib mesylate was kindly provided by Novartis Pharmaceuticals Corporation. All-trans retinoic acid (ATRA) was purchased from Sigma. Chemical structures are shown in Supplementary Fig. 1S.

Quantitative real-time PCR analysis of hTERT expression

Total cellular RNA was collected from samples using TRIzol reagent (Invitrogen) as described by the manufacturer. RNA yields and purity were determined spectrophotometrically at 260 to 280 nm. Reverse transcription reaction was carried out using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer’s instructions with oligo(dT)6 primers. The expression of hTERT transcripts in leukemic cells was quantified by fluorescence real-time PCR using the LightCycler technology and the Light Cycler FastStart DNA MasterPLUS SYBR Green Kit (Roche Diagnostics) according to the manufacturer’s instructions. Primer sequences were 5′-CGGAAAGATGTCTGGAGCAGCAAA-3′ for the sense primer located in exon 3 (hTERT-LCS) and 5′-CTCCCACGACGTAGTCCATG-3′ for the antisense primer located in exon 4 (hTERT-LCR). hTERT level was normalized to the expression of the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH) measured in parallel.

Telomerase activity and telomere length assay

Telomerase activity was measured using the telomerase PCR ELISA kit (Roche Diagnostics) according to the
manufacturer’s instructions. Telomerase activity was expressed as a percentage of that detected in untreated cells. Telomere lengths were determined, after isolation of genomic DNA as described previously (23), using a nonradioactive chemiluminescent TeloTAGGG Telomere Length Assay developed by Roche Diagnostics. The average telomere lengths could be determined by comparing the signals relative to a molecular mass standard.

**Apoptosis assay**

Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technology using In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics), which labeled free 3’-OH DNA cleavage observed during apoptosis. After 3 washes with PBS solution, 2 × 10⁶ cells were fixed using a 4% paraformaldehyde solution in PBS pH 7.4 for 30 min. They were washed again once and could be stored at −20°C during 5 days in 70% ethanol solution or directly permeabilized using 0.1% Triton – 0.1% sodium citrate freshly prepared, for 2 min on ice. After 2 washes, cells were incubated in TUNEL reaction mixture (containing Terminal deoxynucleotidyl transferase which catalyzes polymerization of labeled nucleotides to free 3’OH-DNA ends of DNA strand breaks, mixed to label solution 1:50) in humid atmosphere at 37°C during 1 hour. After 3 final washes, TMR red labeled nucleotides, incorporated in nucleotide polymers, were detected and quantified by flow cytometry.

**Clonogenic assay**

LAMA84-S and LAMA84-R cells were seeded in MethoCult TM H4230 (StemCell Technologies Inc.) at a concentration of 60 and 250 cells/cm², respectively. This semi-solid medium contains methylcellulose (1%), FBS (30%), bovine serum albumin (1%), L-glutamine (2 mmol/L), and 2-mercaptoethanol (10⁻⁴ M). The clonogenic assay was done by adding the cell suspension directly to methylcellulose medium in a 1:10 (v/v) ratio. Finally, the methylcellulose cell suspension was seeded in the dishes and the cultures incubated at 37°C in 5% CO₂ for 20 days. For LAMA84-R cells, imatinib (1 μmol/L) was added to the methylcellulose medium.

**Results and Discussion**

**Imatinib induces a transcriptional repression of hTERT in imatinib-sensitive, not in imatinib-resistant CML cells**

To find new targets and effective strategies for imatinib-resistant cells, we investigated whether telomerase could be involved in the biological effect of imatinib and therefore used a pair of sensitive and resistant blast crisis CML cells.

We first treated the imatinib-sensitive Bcr-Abl positive LAMA84-S cells with increasing concentrations of imatinib (from 0.125 to 1.0 μmol/L) for 48 hours. For each dose tested, we determined hTERT mRNA levels by realtime quantitative PCR, telomerase activity by TRAP assay, and apoptosis by TUNEL assay. Figure 1A shows that treatment with imatinib resulted in a dose-dependent apoptotic response with ED50 between 0.250 and 0.5 μmol/L. hTERT mRNA level and telomerase activity were not significantly modified by imatinib treatment at low doses (0.125–0.25 μmol/L), whereas they were significantly decreased in cells treated at high doses (0.5 and 1.0 μmol/L).

We then did a kinetic analysis to evaluate whether the effects of imatinib at high concentrations on hTERT expression could result from apoptosis induction (Fig. 1B). LAMA84-S cells showed a clear transcriptional repression of hTERT as early as 4 hours after treatment while no significant apoptosis was detected at this time. This effect is clearly not a mere consequence of apoptosis that was clearly detected only after 36 hours of imatinib treatment as telomerase activity decreased.

Imatinib-resistant LAMA84-R cells, maintained continuously under the selective pressure of 1 μmol/L imatinib, showed similar hTERT mRNA levels and telomerase activity (data not shown) as LAMA84-S cells cultured in absence of the drug, even though they displayed shorter telomeres (see below). In contrast to their sensitive counterparts, LAMA84-R cells remained resistant to imatinib-induced repression of hTERT, even at imatinib doses of 2.5 μmol/L (Fig. 1C). These results support a relationship between regulation of hTERT and imatinib sensitivity.

**Imatinib is able to induce apoptosis in imatinib-sensitive LAMA84-S cells through both telomerase-dependent and telomerase-independent mechanisms**

To clarify further whether telomerase was involved or not in imatinib-induced apoptosis, LAMA84-S cells were transduced with hTERT (hTERT-LAMA84-S) and were then sorted on the basis of GFP expression. The expression of the transgene was confirmed both by hTERT mRNA expression and telomerase activity analysis compared to the GFP-control cells (see below). Apoptosis induction by imatinib was then compared in hTERT overexpressing and in mock transduced cells. Figure 2 shows that hTERT overexpression was able to significantly protect LAMA84-S cells from apoptosis induced by 0.250 μmol/L imatinib. In contrast, it did not protect them from the strong and rapid apoptotic effect induced by higher concentrations of this drug. This result indicates that even though imatinib at high concentrations is able to downregulate hTERT expression and activity, apoptosis is likely not the result of this downregulation. Note that the ectopic expression of hTERT and telomerase activity remained unaltered by imatinib treatment (data not shown).

Altogether, these data suggest that imatinib could induce apoptosis through both telomerase-dependent and telomerase-independent mechanisms.

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Long-term ATRA treatment decreases telomerase activity and slows down proliferation of imatinib-resistant LAMA84 cells

Because LAMA84-R cells resist to imatinib-induced apoptosis, we wondered whether targeting hTERT expression could be a means to overcome imatinib resistance. We have previously shown that, in myeloid leukemic cells isolated from patients, ATRA may exert its antiproliferative action independently of differentiation through down-regulation of telomerase activity, which develops slowly during long-term treatment and leads to telomere shortening and subsequent cell death (24). Hence, LAMA84-R cells were incubated in the presence of 1 µmol/L ATRA for a long-term treatment. As shown on Fig. 3A, hTERT mRNA levels measured by quantitative RT-PCR decreased as early as after 3 days of ATRA treatment and remained at 60% of control expression as early as day 7. This decrease was associated with a clear reduction of telomerase activity and a slowdown of proliferation. As a consequence of the decrease of telomerase activity, a small but significant reduction of the mean length of telomeres could be noticed throughout ATRA treatment of the LAMA84-R cells (Fig. 3B). However this decrease of hTERT expression was not associated with increased apoptosis (data not shown). This observation is in agreement with the notion of a threshold determined by the level of enzyme activity, which has to be reached to induce sufficient shortening of telomeres and cell death, as previously reported (25).

The dominant-negative form of hTERT overcomes imatinib resistance in LAMA84-R cells

Even though ATRA treatment slowed cellular proliferation of imatinib-resistant cells, it failed to restore fully imatinib sensitivity, especially as regards cell death induction, because of a limited inhibition of telomerase activity. The fact that ectopic expression of a DN-hTERT variant is able to sensitize K562 CML cells to imatinib-induced cell death has already been established (16,26). Therefore, we...
We then characterized the growth properties of LAMA84-S and LAMA84-R cells expressing either DN-hTERT or WT-hTERT or the control vector. The DN-hTERT transduced LAMA84-R cells exhibited a reduced clonogenic proliferation compared to the control vector or WT-hTERT-transduced cells (Fig. 4, fourth panel). This decrease in proliferation was associated with the induction of apoptosis (data not shown). Altogether, these results show that the repression of telomerase activity by the expression of a dominant-negative form of hTERT was able to restore imatinib sensitivity in an imatinib-resistant cell line. No effect on cell proliferation was observed in DN-hTERT-LAMA84-S cells. It can be speculated that this differential effect of imatinib results from the difference observed in the telomere lengths between the 2 cell lines.

It can be noticed that the antitelomerase strategy through the expression of DN-hTERT was more efficient than ATRA treatment at restoring imatinib sensitivity. As previously pointed out, this can be explained by a higher inhibition of telomerase activity by DN-hTERT than by ATRA, leading to a sufficient telomere shortening and consequently an efficient induction of cell death. This observation supports the notion that telomere length could represent an important parameter to measure in CML patients, especially to evaluate the efficiency of such antitelomerase treatment.

Altogether, these results indicate that the extinction of hTERT expression or activity could alter specifically the viability of imatinib-resistant cells. However, it is important to keep in mind that although LAMA84-R cells were resistant to the toxic action of imatinib, their viability relies on the continuous presence of this drug (as already mentioned, see Materials and Methods). Therefore, it cannot be excluded that the specific action of antitelomerase strategies on imatinib-resistant cells requires cooperation with imatinib signaling pathways.

**hTERT overexpression facilitates imatinib-resistance establishment**

Altogether, the above results indicate that the extinction of hTERT expression or activity could modulate specifically imatinib sensitivity of CML cells and they point to a possible involvement of hTERT in the emergence of imatinib resistance. To check this hypothesis we investigated whether the ectopic expression of WT-hTERT in the LAMA84-S cells could facilitate the development of imatinib resistant clones compared to non-transduced LAMA84-S cells. For this purpose we proceeded as already described for the generation of the imatinib-resistant LAMA84-R cells (18). hTERT-LAMA84-S and their nontransduced LAMA84-S counterpart cells were exposed to successive dose escalations, from 0.125 µmol/L imatinib (first dose-step) to 0.250 µmol/L about 2 weeks later, when cells stopped dying (second dose-step). Proliferation curves (Fig. 5A), cytophin slides (Fig. 5B) stained with May-Grünwald Giemsa
and clonogenic assays (Fig. 5C) clearly showed that hTERT-LAMA84-S cells recovered more rapidly from imatinib-induced cell death than the nontransduced LAMA84-S cells did, allowing a more rapid emergence of resistant clones. Out of the many attempts to generate imatinib-resistant clones, resistant cells emerged reproducibly faster from WT-hTERT-LAMA84-S than from LAMA84-S cells. These observations indicate that hTERT expression is able to give cells a growth advantage during the establishment of imatinib-resistance. The median telomere length of imatinib treated cells shortened during the establishment of the resistance over an expansion time of 15 weeks. In contrast, hTERT expression stabilized telomere length in the hTERT-LAMA84-S cells suggesting that the telomere maintenance function of hTERT is likely to participate in the emergence of resistant cells.

Concluding remarks

Imatinib discovery represents a major breakthrough in the therapy of CML. However, despite satisfactory remission rates, resistance to this drug is an important issue for
therapy, because it is now clear that a significant proportion of patients in advanced disease phases display reduction of imatinib sensitivity over time and undergo relapse. Activation or upregulation of telomerase is believed to play an important role in the progression of most human malignancies. While implication of telomerase in the development of imatinib resistance has already been suggested (27,28), no direct obvious demonstration has been given. Furthermore, some contradictory studies can be found in the literature.

Our results show, first, that imatinib could induce apoptosis in imatinib sensitive cells through both telomerase-dependent and -independent mechanisms; second, that imatinib induces a transcriptional repression of hTERT associated with the induction of apoptosis only in imatinib-sensitive cells and not in their imatinib-resistant counterparts. These observations suggest that telomerase represents an additional factor in imatinib resistance in blast crisis CML cells. This hypothesis has been validated by showing that the emergence of resistance occurred faster in cells overexpressing hTERT compared to control cells. It has been shown that telomerase contributes to installing an immortal cell phenotype by preventing apoptosis, and thus plays a role in cellular resistance to anticancer drugs (12,13,29–31). In keeping with this notion, we showed that hTERT overexpression significantly protects these cells from apoptosis induced by 0.250 μmol/L imatinib. Therefore the emergence of imatinib-resistant cells could result from both the antiapoptotic and telomere-maintenance functions of hTERT.

Hence, targeting this enzyme could be beneficial for the elimination of resistant clones. We therefore submitted imatinib-resistant LAMA84-R cells to 2 antitelomerase strategies known to use different mechanisms: the expression of a dominant-negative form of hTERT and ATRA treatment leading to a transcriptional repression of hTERT. These 2 antitelomerase strategies resulted in either cell death (DN-hTERT transduction) or antiproliferative effects (ATRA treatment). Intriguingly, although constitutive hTERT expression and activity and Bcr-Abl expression did not differ between imatinib-resistant LAMA84-R and imatinib sensitive LAMA84-S cells, these effects were observed only in the imatinib-resistant and not in the imatinib-sensitive cells. It can be speculated that this specific effect on LAMA84-R cells could be partly explained by the fact that telomeres in these cells are shorter than those in the sensitive parental ones and therefore more prone to reach the telomere length threshold ultimately leading to cell death induction. If so, telomere length measurement in newly diagnosed CML patients might have a predictive value for the evaluation of the efficiency of antitelomerase treatments.

Alternatively, because LAMA84-R cells need to be maintained in the continuous presence of imatinib it cannot also be excluded that this specific effect of antitelomerase strategies on imatinib-resistant cells requires cooperation with imatinib signaling pathways.

The complexity of telomerase regulation, which can differ between various cell lines as well as between patient cells, makes it obvious that our treatment protocol, done on a single cell line, could not be effective in all cases. In keeping with this notion, a recent study...
reports that the success of antitelomerase strategies could depend on the presence of a functional p53 pathway (32). Nevertheless, our strategy, i.e., combining antitelomerase agents to imatinib at the beginning of the therapy, should be considered as an attractive approach to be integrated into extended trials to reduce the risk of imatinib resistance development and ultimately increase the probability to eradicate the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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