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TELOMERASE REGULATION IN HEMATOLOGICAL CANCERS: A MATTER OF STEMNESS?

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ABSTRACT

Human telomerase is a nuclear ribonucleoprotein enzyme complex that catalyzes the synthesis and extension of telomeric DNA. This enzyme is highly expressed and active in most malignant tumors while it is usually not or transiently detectable in normal somatic cells, suggesting that it plays an important role in cellular immortalization and tumorigenesis. As most leukemic cells are generally telomerase-positive and have often shortened telomeres, our understanding of how telomerase is deregulated in these diseases could help to define novel therapies targeting the telomere/telomerase complex. Nonetheless, considering that normal hematopoietic stem cells and some of their progeny do express a functional telomerase, it is tempting to consider such an activity in leukemias as a sustained stemness feature and important to understand how telomere length and telomerase activity are regulated in the various forms of leukemias.

1. Introduction

Telomeres [1] are nucleoprotein structures composed of tandem arrays of telomeric repeats (5'-TTAGGG-3'). They preserve genome integrity and prevent chromosome ends from being recognized as double-strand breaks avoiding the activation of DNA damage pathways that result in senescence and cell death. Telomeres of human somatic cells shorten gradually during successive cycles of cell division. This shortening leads to chromosomal instability and senescence.

In contrast to most somatic cells, stem cells, including hematopoietic stem cells (HSCs) and some peripheral blood cells have a significant proliferative capacity that requires telomere length maintenance throughout many divisions. It is achieved in part by the activity of a specialized reverse transcriptase named telomerase which is thought to play an important role in self-renewal of HSCs [2]. However, despite telomerase activity, telomere shortening is not prevented but delayed suggesting that HSCs lack sufficient telomerase activity to completely prevent telomere loss [3]. Because extinguished telomerase expression is a constant feature of functionally mature cells, the question raised by recent works is whether sustained telomerase activity has to be considered as integral feature of stemness. This question is central for cancer cell therapy, but also when considering engineered somatic cell for cell therapy and tissue repair.

Tumor cells including leukemic cells take on properties of undifferentiated or stem cells. These properties result from acquired genetic/epigenetic defects in signaling pathways, making physiological access to maturation, senescence or apoptotic program impossible. Tumor cells, as stem cells, are endowed with self-renewal capacity and have lost the possibility to express a physiological mature and functional phenotype. The current knowledge on telomere and telomerase biology in cancer cells indicates that beside the acquisition of genetic defects, activation of telomerase in these cells constitutes an essential parameter in tumor progression [4-10]. Thus, telomerase/telomere regulation represents a promising target for anticancer drug discovery (reviewed in [11]).

Because the levels of telomerase activity vary in normal hematopoietic cells according to their state of differentiation and activation, the study of telomerase deregulation in hematological malignancies has been a difficult task. The difficulties come from the complex telomerase machinery and its physiological regulation and the multiple cases of regulation evidenced by the distinct types of hematopoietic diseases

This paper will *first* briefly review our current knowledge on telomerase machinery together with an overview of the hierarchy of regulatory mechanisms encountered, and *second*, analyze the deregulation of telomerase in hematological cancers, in order to clarify the feasibility of transferring basic research breakthrough to clinic cases for telomerase-targeted therapeutic strategies.

2. Mechanisms of regulation of telomerase activity

Telomerase is a ribonucleoprotein complex that extends chromosomal ends by the addition of single-stranded TTAGGG repeats [5, 8]. Telomerase consists of two components, a protein reverse transcriptase (hTERT) and a RNA component (hTR), which is used as a template for the elongation of telomeres [3, 12, 13]. hTR belongs to the family of small nucleolar RNAs and contains a H/ACA domain essential for telomerase activity, hTR nuclear accumulation and maturation. Since hTR expression shows a broad tissue distribution, telomerase activity is generally well correlated with hTERT expression suggesting that hTERT is a key regulator of this enzyme. However, it cannot be excluded that the level of hTR can be limiting. Indeed, a recent study showed that overexpression of hTR together with hTERT in cancer cell lines and primary human fibroblasts induced telomerase activity and telomere length elongation to a higher extent than overexpression of hTERT alone showing that hTR may be limiting *in vivo* [14]. Regulation of telomerase operates at several biological levels: transcription, splicing, hTR and hTERT modifications, enzyme trafficking and subcellular localization of each component, assembly in an active ribonucleoprotein, accessibility and action on telomeres [15]. Not only has telomerase a role in the prevention of senescence in highly proliferative cells by compensating for the loss of telomeric ends, which

occurs at each cycle of replication, but it also presents non conventional roles including capping and anti-apoptotic functions, activation of pro-proliferative signaling pathways, modulation of specification of hematopoietic stem/progenitor cells during vertebrate development [16, 17]. Furthermore, emerging evidence indicates that beside controlling replicative lifespan and cell proliferation, telomerase controls cell differentiation through signaling pathways that remain to be unraveled [18-22]. Although most of these extracurricular activities of telomerase have been reported to be independent of its activity on telomere length maintenance, a definitive demonstration is still lacking. Indeed, as in most of these studies only the mean length of telomere was measured, an activity of telomerase on critically short telomeres cannot be excluded.

Telomere structure and functions rely on two different types of complexes, one involves in DNA elongation [23], including hTR, hTERT and dyskerin and one involves in capping, including the shelterin complex [24] (TRF1, TRF2, TIN2, hRAP1, TPP1, POT1). In addition to shelterin, several DNA repair proteins present at telomeres play a direct role in regulating telomere length and protecting telomeres indicating an interplay between telomere function and DNA repair [25]. Removal of telomeric proteins induces a de-protection of telomeric DNA and dysfunction of telomeres leading to their rapid shortening and consequently to cell death.

Regulation of telomerase activity takes place at both telomerase protein and gene levels. The hTERT gene comprises about 35kb DNA and codes for an mRNA comprising 16 exons and 15 introns [26-28]. As activation of hTERT has been shown to be one important limiting step for the induction of telomerase activity, much effort has been focused on the understanding of the transcription regulation of hTERT. However increasing data indicate that regulation of telomerase is a complex process involving many steps.

2.1. Epigenetic regulation of hTERT

The hTERT promoter contains a cluster of CpG sites suggesting its regulation to involve DNA methylation. Some groups showed the association between the methylation status of the hTERT promoter with gene silencing [20, 29, 30]. However, other reports

indicated no significant correlation [29, 31]. Furthermore, contradictory reports have mentioned an increased DNA methylation in hTERT promoter in hTERT-positive cancer cells while lack of methylation was found in normal-negative cells [32]. These apparent discrepancies have been recently solved [33, 34]. In several cancer cell lines and tissues, careful analysis of hTERT methylation patterns has demonstrated a short region of the CpG island located in the hTERT core promoter that remains unmethylated despite highly methylated border regions [33]. Chromatin immunoprecipitation assays have shown that active chromatin marks in this region allow for the expression of hTERT [34] and hTERT methylation in the border region prevents the binding of negatively-acting transcription factors such as CTCF [35]. Recently MBD2 (MethylCpG binding domain protein2) has been shown to be directly involved in transcriptional repression in hTERT-methylated telomerase positive cells [36]. Regulation of hTERT transcription may also involve histone acetylation/deacetylation. Trichostatin A, which inhibits histone deacetylase, activates hTERT gene expression in multiple cell types [37, 38]. However, Trichostatin A may also activate additional genes that directly or indirectly derepress hTERT.

2.2. Transcriptional regulation of hTERT

The transcription of hTERT gene has been suggested to be the key determinant in the regulation of telomerase activity. Transient expression assays using the 3.0 kb of the flanking sequences of the hTERT gene revealed that the transcriptional activity was up-regulated specifically in cancer cells, while it was silent in most normal cells [28]. Deletion analysis of the promoter identified the proximal 260 bp region as the core promoter essential for cancer-specific transcriptional activation [26-28].

A number of binding sites for transcription factors (activators and repressors) have been identified in the hTERT core promoter (reviewed in [39]): two E-boxes (CACGTG located at -165 and +44 from the transcription start site) which are binding sites for basic-helix-loop-helix zipper transcription factors encoded by the Myc family oncogenes (Myc/Mad-1/Max); at least five GC-boxes (GGGCGG), which are binding sites for zinc finger transcription factor Sp1, and various *cis*-regulatory element that may recruit various factors

regulating hTERT transcription (Table 1). However, the participation of many of these factors in hTERT regulation is often deduced from studies based on overexpression and/or gene reporter experiments, which do not mimic physiological situation.

It has been proposed that several human viruses could deregulate telomerase transcription acting in *trans* as transcription factors or in *cis* through viral integration (reviewed in [40]). Indeed, the integration of hepatitis B virus, papilloma virus and more recently avian leucosis virus (ALV) in a small number of liver, cervical tumors and B cell lymphomas have been reported suggesting that proviral integration in TERT may be of general importance in oncogenesis [41-43].

2.3. Post-transcriptional regulation of hTERT

Transcriptional activation of hTERT gene is not necessary the rate-limiting step to generate a functional telomerase. Another regulatory step in controlling telomerase function in cells could result from the differential splicing of hTERT mRNA. Although the regulatory mechanisms underlying these alternative splicing events remain unknown, several groups have found that hTERT mRNA can exist in a variety of splice forms [44-49]. These forms result from complete or partial deletions of exons, or insertion of parts of introns. Alternative splicing could therefore represents a means for cells to modulate telomerase activity by switching their hTERT spliced variant expression profiles during development and in certain tumors [50-57]. Furthermore, some mechanisms of *in vitro* resistance to anti-telomerase strategies could be explained by modifications in the profile of expression of these variants [58]. In the case of complete or partial deletions of exons, hTERT RNA splicing leads to the elimination of important motifs for the reverse transcriptase activity. These variants should then generate truncated protein isoforms deprived of any active role in the maintenance of telomere length. Furthermore, it has been proposed that these splicing products, by competitive interaction with components of the telomerase complex may act as "dominant-negative" isoforms: so far, a dominant-negative function has been described for the α -isoform only [44, 48]. However, it has not been investigated whether some of these variants harbor other functions distinct from telomere maintenance. Beyond what should be

considered today as interesting speculation, the decisive question is to clearly prove that these multiple splice variants isoforms are indeed translated into functional proteins. Today there are only scarce evidences for that, simply because appropriate experimental tools to go further are not yet available (i.e. variant-inducible cell lines, splice variant-specific antibodies, *in cellulo* functional assays for a traceable biological response that develops over weeks...). To face a conceptually important question, investigators are often technically powerless. The answer to this key question will need experimental approaches starting from ground with relevant cellular models.

2.4. Post-translational regulation of hTERT

That it has been demonstrated the presence of apparently full-length hTERT mRNA in samples that lack telomerase activity [59] suggests post-translational regulation of hTERT. Modulation of telomerase activity has been shown in response to a variety of kinase and phosphatase inhibitors [60-63] suggesting telomerase regulation by phosphorylation at specific serine/threonine or tyrosine residues. It remains unclear, however, whether this modulation occurs directly at the level of hTERT phosphorylation itself or alters indirectly telomerase activity. Akt kinase was reported to enhance telomerase activity through phosphorylation of hTERT [60] at Ser 824, whereas c-Abl protein kinase inhibits telomerase activity by tyrosine phosphorylation after interacting with c-Abl SH3 binding domain located in hTERT (amino acid residues 308-316) [61].

2.5. Cellular localization of telomerase

Another important level of telomerase activity regulation is the proper assembly of hTERT with other telomerase components and proper localization of this complex to at least one of its sites of action, the telomeres [64, 65]. The assembly of active telomerase involves the stabilization and transcriptional upregulation of hTR and its functional association with hTERT. Processing and stability of hTR require binding of nucleolar RNA binding proteins (H/ACA proteins) such as dyskerin, NHP2, NOP10, and GAR1 to the H/ACA motif [66]. Subcellular transport of hTERT has also important implications in its functional regulations. hTERT has been localized in the nucleus [67]. Yang *et al* observed that hTERT protein could

either be concentrated or excluded of the nucleolus within the nucleus [68]. In another study, both TERT and TR have been found to localize to nucleolus, leading to the suggestion of nucleolus as the site for telomerase ribonucleoprotein complex biogenesis in human cells [66]. Furthermore, it was reported that hTERT underwent a dynamic subnuclear shuttling between nucleolus and nucleoplasm that was dependent on the cell cycle, DNA damage or transformation [69-72]. However, this result has been recently challenged by studies based on mutational inactivation of a nucleolar-targeting signal that showed that although TERT mutant did not localize in the nucleolus, it still had telomerase activity, could maintain telomere length and did not affect cellular proliferation [73]. This study suggests that nucleolar localization is not related to telomerase canonical functions.

hTR was recently shown to accumulate in Cajal bodies, subnuclear structures implicated in ribonucleoprotein maturation [70, 74]. This localization is specific to cancer cells where telomerase is active and seems dependent on hTERT although this point remains controversial [72, 74]. Furthermore, during S phase both hTR and hTERT colocalize within Cajal bodies that are associated with telomeres [71, 75]. During the other phases of the cell cycle, hTERT is not detected in the Cajal bodies. These studies are very important as they suggested that Cajal bodies, serving as sites of telomerase maturation and assembly to deliver active telomerase to telomeres might be a key factor in telomere length homeostasis. The assembly of the telomerase holoenzyme is controlled by chaperone proteins helping the formation of active enzyme [76]. The Hsp90 and p23 proteins remain permanently associated to the telomerase complex affecting its activity.

Beside this nuclear localization of telomerase, it has recently been demonstrated that the N terminus of hTERT has a mitochondrial leader sequence and that active telomerase can be detected in mitochondrial extracts of hTERT overexpressing cells. Therefore telomerase has been added to a growing list of proteins that can shuttle between the nucleus and different subcellular compartments including mitochondria [77, 78]. However the biological significance of hTERT translocation into mitochondria is still elusive and controversial.

The above observations indicate that further investigation is still required to explore the importance of hTERT cellular localization. Moreover, cautious has to be taken regarding the already published results as some of them were mainly deduced from experiments using ectopic expression of hTERT while in others direct detection of hTERT has been performed with antibodies with low specificity if any. Indeed, it has been recently unambiguously demonstrated that one of the most frequently used anti-hTERT antibody, the NCL-hTERT from Novocastra antibody in fact recognized nucleolin rather than telomerase [79]. The conclusions previously drawn from studies using this antibody began to be reconsidered [53, 80].

2.6. Telomerase regulation by telomeric proteins and RNAs

Mammalian telomeres have been observed in a special conformation, called t-loop in which the telomere terminus is unlikely to be accessible to telomerase. This structure is associated with telomeric binding proteins including those of the shelterin complex which play an important role in the regulation of telomere length as already mentioned. TRF1 and TRF2 directly bind to the double-strand telomere repeats. TRF1 is a negative regulator of telomere length [81] and TRF2 is essential for telomere protection and promotion of the t-loop [82]. POT1 which binds the single-stranded telomeric DNA transduces the information sensed by TRF1 to telomerase resulting in elongation of critically short telomere [83]. POT1 and TPP1 can form a complex with telomeric DNA that has recently been shown to increase the activity and processivity of telomerase [84]. It has also been shown that PARP-1 modulates telomerase activity by altering poly-ADP-ribosylation of hTERT and/or the expression of telomerase associated protein1 (TEP1/TP1) subunit of telomerase holoenzyme [85]. All these observations show that having an understanding of how telomeric proteins levels vary in tumors is fundamental for clinical studies and may help to predict the success of treatment with telomere-targeted agents. Finally, the newly discovered telomeric RNAs transcribed from telomeric repeats by DNA-dependent RNA polymerase II, TelRNAs or TERRA [86, 87], block the activity of telomerase *in vitro*, possibly through pairing with the

template region of hTR, suggesting that TelRNAs may regulate telomerase activity at chromosome ends.

3. Deregulation of telomerase in hematological cancers

Several factors are important to consider for interpretation of telomerase expression and activity levels in leukemias such as variability among individuals and heterogeneity at the level of the cell populations that are analyzed. The role and the mechanisms of reactivation of telomerase in leukemic development remain currently under debate. Telomerase positivity may occur by expansion of a pre-existing telomerase-competent clone or alternatively by upregulation in previously telomerase-negative cells as a consequence of genetic instability or acquired genetic defects.

When considering hematological cancers one has first to remind the key feature of the developmental process of normal hemopoiesis: a stem cell-based system, where hemopoietic stem cells have an extraordinary capacity of self-renewal. However, this potential is fully exploited during pathological situations, when the stem cell reservoir has to be rapidly mobilized according to the demand of a fast differentiating cell compartment. The steady daily hemopoiesis is in charge of committed progenitors whose cell fate is aimed at the production of short-life mature cells. This differentiating cell compartment is exceptionally rich in cell phenotypes which when matured have to achieve multiple biological functions (anti-bacterial, anti-viral, anti-parasite immune responses, survey of the production of red blood cells, phagocytes involved in cell defense and tissue clearance of apoptotic cells, coagulation, etc...). Each of these mature cell types exhibits a specific physiology, and requires a specific homeostatic control of the functional population. The gain of specialized functions is acquired at the expense of the proliferation potential. Senescence and elimination of injured or aged cells by phagocytes, and programmed cell death altogether contribute to avoid potentially oncogenic events to be settled by the proliferation bursts necessary to the clonal production of specialized hematopoietic cells. It is highly intriguing that telomerase whose expression is observed in hematopoietic stem cells, progressively

decreases along differentiation of hematopoietic progenitor cells and is annihilated in short-life mature cells. The intimate link between telomerase expression in the hemopoietic system and the hierarchy cells with distinct self-renewal potential observed during the developmental process, led to consider telomerase expression and function as a matter of stemness.

Recent data suggest that telomere dysfunction can induce environmental defects that impair the function and engraftment of hematopoietic stem and progenitor cells [88, 89]. This finding has implications for therapy as telomere dysfunction could limit the therapeutic potential of transplanted cells and also could contribute to the increase cancer initiation that has been observed in aging telomere-dysfunctional mice [90]. Indeed, the loss of telomere functions resulting from either telomerase disorder or loss of hematopoietic stem cells can provide selection for abnormal cells and result in malignant progression facilitated by genomic instability due to uncapped telomeres.

Without maintenance of telomerase activity and thus telomere maintenance, the oncogenic transformation of either myeloid stem cells (ref. CML) or committed progenitors (ref. AML), would have little probability to settle as an hematopoietic cancer.

Therefore, the questions as to whether 1) telomerase expression and dysregulation take part in the etiology of hematopoietic cancers, 2) telomerase downregulation mechanism constitutes a protection against oncogenic events, and 3) telomerase can be a therapeutic target, cannot be eluded. These questions can only be considered, case by case, within each pathology context.

3.1. Myelodysplasia and Acute Leukemia

Several studies have investigated telomerase activity and telomere length in total mononuclear cell populations from patients with Myelodysplasia (MDS) and Acute Myeloid Leukemia (AML) [91, 92]. Hematopoietic cells from MDS patients express low level of telomerase [93]. This means that telomere erosion due to rapid cell division may not be prevented in most the MDS cells. This loss of telomere stability may induce further genetic abnormalities leading to other mutations and disease progression. MDS is a preleukemic state with approximately 25% of patients progressing into acute myeloid leukemia. Incidence

of leukemic transformation is significantly higher in MDS patients with shortened telomeres. Therefore, short telomeres without elevated telomerase activity may be a hallmark of MDS cells and of importance in the pathogenesis of the disease. AML with multiple chromosome aberrations are characterized by critically short telomeres [94, 95] but high telomerase activity. A recent study performed on 40 patients during the course of acute promyelocytic leukemia (APL) disease showed that shortened telomere length and elevated telomerase activity correlate with disease progression and relapse, and that this parameter may serve as prognostic factors for a subset of APL patients with more aggressive disease who may not respond favorably to therapy [96].

3.2. Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML), a myeloid stem cell disease, represents a model disorder to study the role of telomere/telomerase biology for disease progression in a malignant stem cell disorder. This disease is a clonal myeloproliferative disorder characterized by the Philadelphia (Ph) chromosome, due to a reciprocal translocation involving the long arms of chromosomes 9 and 22. This translocation generates a fusion gene, BCR-ABL, which encodes a chimeric p210^{BCR/ABL} protein tyrosine kinase that is necessary and sufficient for cell transformation [97]. CML is characterized by distinct clinical phases: a relatively stable chronic phase (CP) that can last for many years, in which mature granulocytes are still produced even though patients have an increased number of myeloid progenitor cells in the peripheral blood, followed by an accelerated phase (AP). The AP, associated with increased genetic instability and acquisition of additional cytogenetic abnormalities, is followed by a blast crisis (BC), in which hematopoietic differentiation has become arrested. The availability of imatinib mesylate, a BCR-ABL tyrosine kinase inhibitor, has revolutionized the treatment of CML [98].

T. Brummendorf *et al* found that telomeres in Ph(+) cells are significantly shorter than in Ph(-) cells from the same individuals [99]. In line with this finding, cytogenetic and molecular response after imatinib therapy are correlated with an increase in mean telomere length [100].

Several published studies demonstrate changes of telomere length and telomerase activity between CP and BC. Telomerase activity has been shown to increase in the bone marrow cells of patients with CML during disease progression [101-104]. Telomere length is generally shorter in AP and BC compared with serial samples taken at CP. Since the levels of telomerase activity is elevated but not enough to maintain telomere length, telomere length reflects a proliferative history of leukemia cells closely related to disease severity. Furthermore, it has been shown that telomere shortening observed at the time of diagnosis significantly influences the time of progression to AP [99, 105].

However, in other studies, it has been shown that hTERT is downregulated in the purified CML CD34⁺ cells relative to the normal CD34⁺ cell population and that gene expression levels of hTERT progressively decrease with disease evolution [106]. Since previous studies have been performed mostly on unfractionated bone marrow samples, this discrepancy might simply reflect the differences in the number of immature cells present in the sample: mononuclear fraction in BC CML contains higher numbers of blast cells than mononuclear fraction from normal individuals or CP CML patients. In support of these results Drummond et al. showed a lack of overexpression of hTERT as well as reduction of hTR expression levels in CD34⁺ cells of CP patients compared to controls [52]. These results suggest that the increase in telomerase activity observed in patients might actually only reflect the increased proportion of cycling cells in bone marrow [52] and not a genuine increase in telomerase activity. However, it has to be kept in mind that the CD34⁺ cells, immuno-selected strictly on expression of this marker, are not identical in all biological aspects in patient and control patients. The link between telomerase expression and the progenitor status might prove more subtle than initially thought. Finally, altered mRNA splicing appeared to play a significant role in determining functional hTERT levels and telomere maintenance during the CP of CML and may influence the disease progression [52] but this issue has to be analyzed in consideration of the difficulties linked to approach the biology of hTERT splice variant isoforms, as discussed above.

3.3. T-cell leukemia/lymphoma

The oncogenic human retrovirus HTLV-1 (human T-cell leukemia virus type I) has been identified as the etiologic agent of adult T-cell leukemia/lymphoma (ATLL), a lymphoproliferative disorder of infected CD4+ T-cells. Several lines of evidence established that HTLV-1 Tax protein play a central role in the conversion of normal T cell to a leukemic cell. Similar to the pattern seen in most leukemia, telomeres are short in HTLV-1 infected cells despite the presence of strong telomerase activity [107-110]. The reactivation of telomerase seems to be a key event in development and progression of ATLL. High telomerase activity and shortened telomere length were associated with poorer prognosis, indicating that these parameters may be useful markers for predicting the course of disease [110].

The effect of Tax protein on expression of telomerase activity in human cells remains controversial. One study suggested that the HTLV-1 encoded Tax protein had a negative effect on hTERT promoter reporter vector in transient assays [111] even though this observation is difficult to reconcile with the capacity of this protein to immortalize T-cells. However, this repression was observed when Tax-expressing cells were subjected to mitogenic stimulation. This result has been challenged by recent data indicating, in contrast, that in the absence of mitogenic stimulation, Tax stimulates the endogenous hTERT promoter through the nuclear factor κ B (NF- κ B) signaling pathway mediated by Sp1 and c-Myc [112]. This discrepancy can be explained by differences in the cell systems used but also by a cell cycle dependent effect of Tax on hTERT expression. Indeed, a recent paper showed that Tax activates hTERT promoter only in quiescent T-cells and not in growing T-cells [113]. Recently, other alternative mechanisms for telomerase activation independent of the viral Tax protein have been identified [40, 114]. One involves the HBZ (HTLV-1, bZIP factor) identified in ATLL cells that can be responsible for the increase of hTERT transcription during late stages of leukemogenesis. The other one is dependent on the presence of interleukin-2 (IL-2) and involves the PI3K/Akt pathway. Furthermore, it has been demonstrated that IL-2 signaling was associated with PI3K dependent/Akt independent transcriptional up regulation of endogenous hTERT promoter. The activation of PI3K

pathway mediated cytoplasmic retention of the Wilms tumor (WT1) protein, which strongly suppressed hTERT promoter. This model provides an explanation for the elevated telomerase activity in IL-2 dependent cells and ATLL patient samples even in the absence of the HTLV1-Tax protein. It has been reported that telomere lengths were significantly shorter in ATLL cells, despite expressing high telomerase activity, compared to non infected cell isolated from the same patients [108]. This shortening of telomeres has been associated with an increased expression of telomere binding proteins TRF1, TRF2 and TIN2 which may increase genetic instability and tumor progression. These results suggest that these genes could constitute potential target for future ATLL therapy.

3.4. B-cell malignancies

B-cell chronic lymphocytic leukemia (B-CLL) results from the progressive accumulation of a leukemia clone [115]. The identification of reliable prognostic markers is essential in this leukemia, which is clinically heterogeneous. Many reports provide evidence that telomere length as well as telomerase activity exerts a strong impact on the survival of B-CLL patients. A low telomerase activity is observed at disease onset [116] and increased activity in advanced stages and bad prognosis groups [117]. Telomeres are shorter in B-CLL cells versus normal B cells and especially short in patients with bad prognosis. These observations make that telomerase activity and telomere length can be used as new prognostic markers in this disease. A recent transcriptome analysis of the telomere components, the shelterin proteins and multifactorial proteins involved in telomere maintenance shows both telomerase downregulation and changes in telomeric protein compositions in peripheral B cells from 42-B-CLL patients. However these changes have to be confirmed at the protein level [118].

A relevant role of Epstein-Barr virus (EBV) in the pathogenesis of B-cell malignancies, including post-transplant lymphoproliferative disorders and immunoblastic lymphoma, is strongly suggested. Transformation of primary B-lymphocytes by EBV requires the expression of several latent viral proteins that include six nuclear antigens (EBNAs) and three latent membrane proteins (LMP1, LMP2A, LMP2B). Among them LMP1 is considered

as the strongest oncoprotein, being essential for B cells immortalization. It has been demonstrated that activation of hTERT was associated with the progressive increase of LMP1 expression and inhibition of viral lytic replication [119] favoring the induction and latency in B lymphocytes. The contribution of hTERT to EBV-driven B cell immortalization resulted not only from preventing telomere erosion but also from playing a crucial role in the establishment and maintenance of a latent infection. Although the molecular mechanisms underlying the interplay between EBV and hTERT need further investigation, these results support the notion that hTERT may constitute a relevant therapeutic target for EBV-associated B cell lymphomas.

4. Antitelomerase strategies for leukemia

In most hematological cancers, telomere lengths are generally shortened and telomerase deregulated. These observations are indications that telomere length and telomerase expression and activity can serve as molecular markers of the clinical progression and prognosis of most leukemia. Furthermore, they raise the possibility that anti-telomerase strategies may provide novel approaches in the treatment of leukemia.

Since telomerase and telomere maintenance play a key role in hematological malignancies, several approaches can be developed. The telomerase protein complex offers multiple potential inhibitory sites, including hTR and hTERT at both transcriptional and post-transcriptional levels. Furthermore, strategies targeting telomeres as well as approaches targeting telomerase positive cells have also been developed. They have already been described [11] and are summarized in Table 2. Some of these antitelomerase strategies proved to be promising in some types of leukemia. They include both specific compounds designed to target specifically the components of telomerase/telomeres and already clinically relevant drugs with telomerase-modulating properties. They will be developed below.

4.1. Drugs targeting specifically telomere/telomerase

The small molecule BIBR1532 (2-[(E)-3 naphthalen-2-yl-but-2 enoylamino]-benzoic acid), a selective, non-nucleosidic inhibitor of the catalytic component hTERT has been

shown to have direct cytotoxic effect against primary leukemic cells from AML, CML, and CLL patients but not against normal hematopoietic stem cells [120]. In addition to telomerase inhibition, this compound can exert a direct cytotoxic effect by compromising the binding of protein partners of telomerase and telomeres resulting in their dysfunction. However, despite initially promising this compound has not progressed into clinical trials.

The use of specific ligands leading to G quadruplex telomeric structure stabilization appears as a promising area of development as they produce an anticancer effect by inhibiting the capping and catalytic functions of telomerase and inducing the dysfunction of telomeres by destabilizing the shelterin [121, 122]. A subset of them has been investigated *in vitro* on leukemic cell lines. Among them telomestatin has been tested in acute leukemia. Decreased tumor telomerase levels, reduced tumor volumes and marked apoptosis in tumor tissue have been observed after systemic intraperitoneal administration of this agent in U937 xenografts [123]. BRACO-19 is a 3, 6, 9-trisubstituted acridine compound which is shown to produce short- and long-term growth arrest in cancer cell lines and to reduce telomerase activity leading to long-term telomere length attrition [124, 125]. RHSP4 is a pentacyclic acridine that binds G quadruplex DNA and inhibits telomerase activity at submicromolar levels [126, 127]. Long-term exposure of cells to low RHSP4 concentration causes an irreversible growth arrest and telomere erosion demonstrating that this compound possesses classical telomerase inhibitory properties. However, used at higher doses, RHSP4 triggers short-term apoptosis or senescence without telomere shortening. This effect suggests that this compound is more than a simple telomerase inhibitor. The short-term effect results from telomere dysfunction [128]. This effect appears to be selective on transformed and cancer cells as RHSP4 appears safe on normal cells. These findings validate telomere as promising targets for future anticancer therapies and open the door to future clinical development of this new class of antitumor agents.

A telomere substrate antagonist, GRN163 an oligonucleotide complementary to the telomerase RNA, and its lipid-conjugated derivative GRN163L, inhibit telomerase activity and

are active against multiple myeloma and lymphoma [129, 130]. GRN163L enters into clinical trials in chronic lymphocytic leukemia and multiple myeloma.

Human hTERT specific epitopes are expressed on cancer cells and not on normal cells. Owing to its expression in almost all cancers, telomerase can thus be considered as a potential molecular target for immunotherapy. Several therapeutic vaccines offer the potential to stimulate the rapid killing of tumor cells by enhancing the activity of telomerase specific cytotoxic T cells [131]. Trials have been initiated in several types of solid tumors and in AML. To date no significant toxicity to normal tissues has been seen.

4.2. Already clinically relevant drugs

Since complete remission has been described in acute ATLL patients after receiving a combination therapy including interferon (IFN) and a reverse transcriptase inhibitor, azidothymidine (AZT) [132], it can be suggested that drugs designed to target the reverse transcriptase activity of hTERT may be effective for the treatment of ATLL.

Among acute myeloid leukemia, APL was found to be particularly sensitive to differentiation therapy using ATRA (*all-trans* retinoic acid). This drug induces remission of APL patients by stimulating differentiation of leukemic cells [133, 134] and is currently used in clinic. In our laboratory, using the NB4 cell model for APL pathology [135], we showed that ATRA not only transcriptionally repressed hTERT in differentiation-associated pathway, as already demonstrated in myeloid HL60 cells [136] but also independently of differentiation. Indeed, in a maturation-resistant NB4-LR1 cells, we showed that ATRA exerts an anti-proliferative activity through hTERT downregulation, which leads to telomere shortening and cell death [137]. It requires the co-activation of the retinoic acid receptor alpha (RAR alpha) and the retinoic X receptor (RXR) [138]. These results have been confirmed also in cells, not only from patients with APL but also with other myeloid leukemia including CML [139]. These findings are of importance for therapy of APL but also of other leukemias because they show that a telomerase-dependent mechanism can be targeted by retinoids in maturation-resistant cells. However, it has been also shown that resistance could emerge from such a telomerase-based therapy [140]. Interestingly, this resistance can be relieved by the

synergistic effect of ATRA/Arsenic trioxide, a treatment that triggers downregulation of hTERT followed by telomere shortening and subsequent cell death [141]. This observation could explain at least partly the success of this combination therapy in APL patients. In contrast with other antitelomerase strategies that are currently developed (see Table 2), this telomerase based antitumor response operates at the level of hTERT gene transcription, therefore possibly targeting more than one telomerase function.

Given the pathogenesis of CML, telomerase inhibition may represent an attractive therapeutic approach. Recently the introduction of imatinib mesylate (Gleevec), an inhibitor of the BCR-ABL tyrosine kinase activity, has strongly improved the therapy of CML leading to significant hematological and cytogenetic remissions and to the increase of the mean survival time [97]. One study has demonstrated that the ectopic expression of dominant negative hTERT in K562 cells enhanced apoptosis of cells compared to control treated with imatinib [142]. It would be interesting to determine if in imatinib resistant cell lines the expression of this hTERT isoforms could overpass imatinib resistance. Furthermore, results obtained in cell lines, although they do not allow to tell if the effect on telomerase was direct or not, suggest that hTERT overexpression may be associated with a partial escape from imatinib-induced apoptosis and that resistance may be mediated via telomerase activation [143]. Interestingly, long-term inhibition of BCR-ABL/ABL synthesis by antisens induced telomerase activity and telomere lengthening [144]. It is therefore not excluded that this activation can be partly responsible for the development of resistance to BCR-ABL drugs even though this issue has never been explored. Taken together, the role of telomerase expression in disease progression, and imatinib resistance in CML, including analysis of telomeres requires further investigation.

5. Conclusion

Given that deregulation of hTERT expression seems to be a general mechanism in leukemia it is worth considering treatments targeting telomere/ telomerase biology. However,

while many advances in telomere/telomerase biology have been uncovered and many efforts have been done to discover new anti-telomere/anti-telomerase drugs, very few compounds are to date exploitable in clinic for reasons of low effectiveness or toxicity *in vivo* and also because of our partial knowledge on telomere and telomerase regulation and functions. It is also becoming clear that focusing solely on a relative inhibition of telomerase activity may misjudge the therapeutic efficiency of some telomerase inhibitors. Indeed, it has been reported in some case that a very high inhibition of telomerase activity or expression is needed to affect telomere shortening and cell viability [145]. This requirement is not always fulfilled. This feature has to be taken into account for the development of telomerase inhibitors. Furthermore, the complexity of telomerase regulation and malignant transformation, as well as the variation in the clinical response from patient to patient, show clearly that the use of a single agent could not be of therapeutic benefit. Consequently an attractive strategy would be to associate, in combinatorial treatments, both conventional chemotherapy and specific drugs targeting telomere/ telomerase biology. Furthermore, it would be also relevant to investigate whether clinically approved drugs can also influence telomerase in a convergent manner. A theoretical disadvantage of telomerase-based strategies lies in the presence of telomerase activity in stem cells and germ cells and also in the identification of very low levels of telomerase activity in somatic cells raising the possibility of side effects. This is why investigation of telomerase expression and regulation during a stem cell development process like hemopoiesis can definitely help to determine the conditions for a physiological regulation of telomerase, exploiting phenotypic variations between differentiation stages and cell lineages. Hemopoietic tumor cells, particularly in the APL cells model, have proved to encompass enough phenotypic plasticity to make these subtle differences therapeutically exploitable. Finally, as it now emerges that telomerase plays additional roles independent of telomere maintenance, like anti-apoptotic functions possibly involved in cancer drug resistance, the clarification of these telomerase functions in hematopoietic cells is becoming an urgent task.

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Table 1: Transcriptional and non-transcriptional factors controlling the expression of hTERT

Regulators	Impact on hTERT expression	Mode of action	References
Transcription factors			
c-Fos/c-Jun/JunD	-	Direct	[146]
c-Myc	+	Direct	[147, 148]
Mad-1	-	Direct	[149-152]
Sp1	+	Direct	[151, 153]
USF-1/2	-	Direct	[154]
	+	Direct	[155]
WT1	-	Direct	[156]
Ets	+	Direct	[157]
	-	Direct	[158]
HIF-1	+	Direct	[159, 160]
	-	c-Myc	[161]
NF-KB	+	Direct	[162, 163]
E2F-1	+	Direct	[164]
	-	Sp1	[165]
P53	-	Sp1	[166, 167]
	-	P21/E2F	[168]
P73	-	c-Myc/Sp1	[169]
Rb	-	E2F-1/cdk2/cdk4	[170]
Hormones			
Estrogen	+	c-Myc	[171]
	-	Direct	[172, 173]
Progesterone	+	MAP kinase	[174]
	-		
Other factors			
TGF β	-	Smad3/c-Myc	[175-177]
Bmi-1	+	P16	[178, 179]
IRF-1	-	?	[180]
P16 ^{INK4a}	-	Sp1	[181]
P27 ^{KIP1}	-	cMyc/Sp1	[182]
	-	IFN γ /IFR-1	[183]
Survivin	+	c-Myc/Sp1	[184]
HPV E6	+	c-Myc/Sp1	[185, 186]

Table 2: Antitelomerase strategies

Therapeutic strategies	Name of the drug	Applications tested	Type of cells	References
Oligonucleotides template antagonist	GRN163/ GRN163L	Myeloma cells	INA6, ARP, MM1S, OPM1, xenografts	[187]
		Renal and prostatic cells lines	Caki-1, DU145	[188]
Hammerhead ribozymes		Human and mouse cancer cells	MCF-7	[189]
		Ovarian cancer cells		[190]
		Breast tumor cells		[191]
hTERT mutant	Dominant Negative -hTERT	CML	K562, OM9;22	[142, 192]
		ALL	HAL-01	[193]
		AML	primary cells of AML patients	[192]
Stabilizing G-quadruplex	Telomestatine (STO-095)	Human Leukemic Monocyte lymphoma	U937	[194]
		AML	NB4, U937	[123, 194]
		Multiple myeloma	ARD, ARP, MM1S	[195]
		CML	K562	[196]
	BRACO-19	Uterus carcinoma cell line	UXF1138L	[124, 125]
RHSP4	Breast tumor cells, Vulva tumor cells	21NT, A431, xenografts	[126-128]	
Immunotherapy:vaccines targeting telomerase	(GRNVAC1, GV1001, p540-548,Vx01)	Breast, prostate, pancreas, kidney, colon, lung	Patients with advanced or metastatic cancers	[131, 197]
Small molecules	AZT (3-azido-2',3'-dideoxythymidine)	Adult T-cell lymphoma	HTLV-1 cell line, Patients	[198]
		B and T lymphoma	JY616, Jurkat E6-1	[199]
	BIBR 1532	AML, CCL, CML	HL60, JVM13, Nalm1	[120]
		AML, CML	Patient cells	[120]
Differentiation-inducing agents	ATRA	APL	Patients	[139]
		CML	Patients	[139]
	9cUAB30	AML	HL60	[200]
	As2O3	APL	NB4	[141]