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QUARTERLY

Minireview

Telomerase as a therapeutic target*

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Ribonucleoprotein telomerase is an enzyme that elongates telomeric DNA. In cells without detectable telomerase activity telomeres shorten with every cell generation and reaching critical length is a signal for cell death. Normal human somatic cells express undetectable, or low (bone marrow and peripheral leukocytes), telomerase activity. Reactivation of telomerase (immortalization) is probably necessary during development of a fully malignant cancer. Consequently, telomerase was proposed to be a therapeutic target for the cancer therapy. Potential results (including side-effects) of telomerase inhibition are being considered. After all, telomerase inhibition can be useful not only in the therapy, but also in cancer biology research, elucidating ageing and immortalization phenomena.

Eukaryotic chromosomes terminate with telomeres — a variable number of short species-specific telomeric repeats [1]. In mammals the sequence: TTAGGG (strand oriented 5′→3′ toward the chromosome terminus) is repeated. Telomeres appear to function in: chromosome stability — protection from DNA degradation, the end-to-end fusion and recombinations; chromosome positioning — attachment to the nuclear membrane; and replication. Telomeres are believed to form specialised structures, and interact with a number of associated proteins. Enzymes that elongate telomeric DNA (telomerases) are ribonucleoproteins [2]. They consist of an essential RNA and a few proteins. RNA contains a region complementary to telomeric repeats. It has been proved that these sequence serves as a template during synthesis of telomeric DNA. Therefore, telomerase is a specialised reverse transcriptase with the built-in template rather than a terminal transferase.

CELLULAR REPLICATIVE SENESCENCE

DNA-dependent DNA polymerases are unable to fully replicate ends of linear DNA (because they can synthesise DNA only in the 5′ → 3′ direction and require an RNA primer). Telomeres can be also lost by degradation by exonucleases. Thus, telomeres shorten in cells lacking telomere elongation activity. According to the telomere theory of cellular ageing, shortening of telomeres acts as a mitotic clock, responsible for cellular replicative senescence [3]. Normal human somatic cells express undetectable, or low (peripheral and bone marrow leukocytes) telomerase activity. Consequently, their telomeres shorten by 50–200 base-pairs with every cell generation. Reaching of critical length (after about eighty divisions for cells derived from a new-born child) is a signal for

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Abbreviations: TRAP, telomeric repeat amplification protocol.

finishing proliferation and afterwards cell death. The exact molecular mechanism of this proliferation crisis is not known. Both transcriptional activation — caused by changes in subtelomeric chromatin structure, and silencing of subtelomeric genes is possible. Silencing could result from changes in subtelomeric chromatin structure, or lack of enough space for transcriptional machinery between the point where a chromosome is anchored to the nuclear cage, and promoter/enhancer regions. Simple excision seems to be unlikely, since cells reaching the replicative limit (Hayflick's deadline) still have some telomeric DNA left.

There is some evidence that the so-called proliferational crisis consists of two stages. The first barrier, called the mortality stage 1 (M1), is associated with activation of p53 and retinoblastoma (RB) cycle-inhibiting genes, somehow induced by shortened telomeres. M1 can be bypassed by transformation with some oncogenes (traditionally called: immortalising oncogenes) for example SV-40 T-antigen. This is not, however, real immortalization. Transformed cells are still telomerase activity negative and show the telomeric decline. Eventually, they reach a second barrier called the mortality stage 2 (M2). M2 means that telomeres are physically too short to fulfil their functions any longer. Numerous chromosome aberrations, especially chromosome fusions, can be observed. Cells cannot divide; they will finally die unless telomerase is reactivated.

Unlike somatic cells, human germ-line cells express a high level of telomerase activity. This is necessary to ensure that telomeres will not shorten in next generations, leading to extinction of the species.

What we do not know is whether cellular replicative senescence is related to ageing of a multicellular organism. About eighty cell divisions, available for human cells, seem to be enough for a lifetime. Cells derived from very old individuals still can divide *in vitro*. What is more, rodents age although their somatic cells mostly express telomerase activity (however, their telomeres still generally shorten). On the other hand, premature shortening of telomeres was found in progeria (human disease associated with early ageing) [4]. However, accelerated accumulation of oxidised proteins was also found in progeria, suggesting rather oxidative than replicative senescence.

There is no doubt that cellular replicative senescence is a mechanism protecting against malignancy. One should remember that human cells are relatively resistant to transformation, both *in vitro* and *in vivo*. A single cell which starts to divide constantly (due to the activation of oncogenes and inactivation of antioncogenes) is a "mother" of malignancy. All cancer cells observed in the advanced disease are the progeny of the "mother" cell. Such a great number of divisions would be impossible without telomerase activity. Therefore, we can consider the reactivation of telomerase to be prerequisite for the development of malignancy in humans [5]. In the case of carcinogenesis *in vitro*, the telomerase recovery is probably responsible for overcoming the proliferation crisis of transformed cells (Fig. 1) [6, 7]. This is similar to carcinogenesis *in vivo*, since telomerase is not active in benign solid tumours (e.g. benign adenomas of colon, uterine fibroids and prostatic hyperplasia), and early leukaemias (e.g. acute myeloid leukaemia and chronic lymphocytic leukaemia), but its activity is detectable in solid malignant tumours and in advanced leukaemias [5, 8–10] (Fig. 1). When these findings are combined with the telomere theory of cellular ageing, we can see, that the step of immortalization (induced by the reactivation of telomerase, probably due to a mutation of its repressor) is necessary for the development of malignancy. This is strongly supported by the fact that immortalised human cell lines, or cell lines derived from rodents are much more susceptible to transformation *in vitro* [11].

There are, of course, facts which cannot be simply explained and seem to be exceptions from this attractive comprehensive theory. There are rare examples of malignant tumours without telomerase activity. It may be that cells of these cancers are not really immortal, but are able to spread and be harmful within their normal replicative life-span. It is also possible that these cells use another mechanism to elongate their telomeres. They could, for example, do it through the recombination-based pathway similar to that observed in yeast cells.

It is generally difficult to establish cultures of cancer cells *in vitro*. Why is it so if these cells are immortal? Early studies failed to demonstrate telomerase activity in fresh cancer samples. Now, with the new PCR-based assay: the telomeric repeat amplification protocol (TRAP),

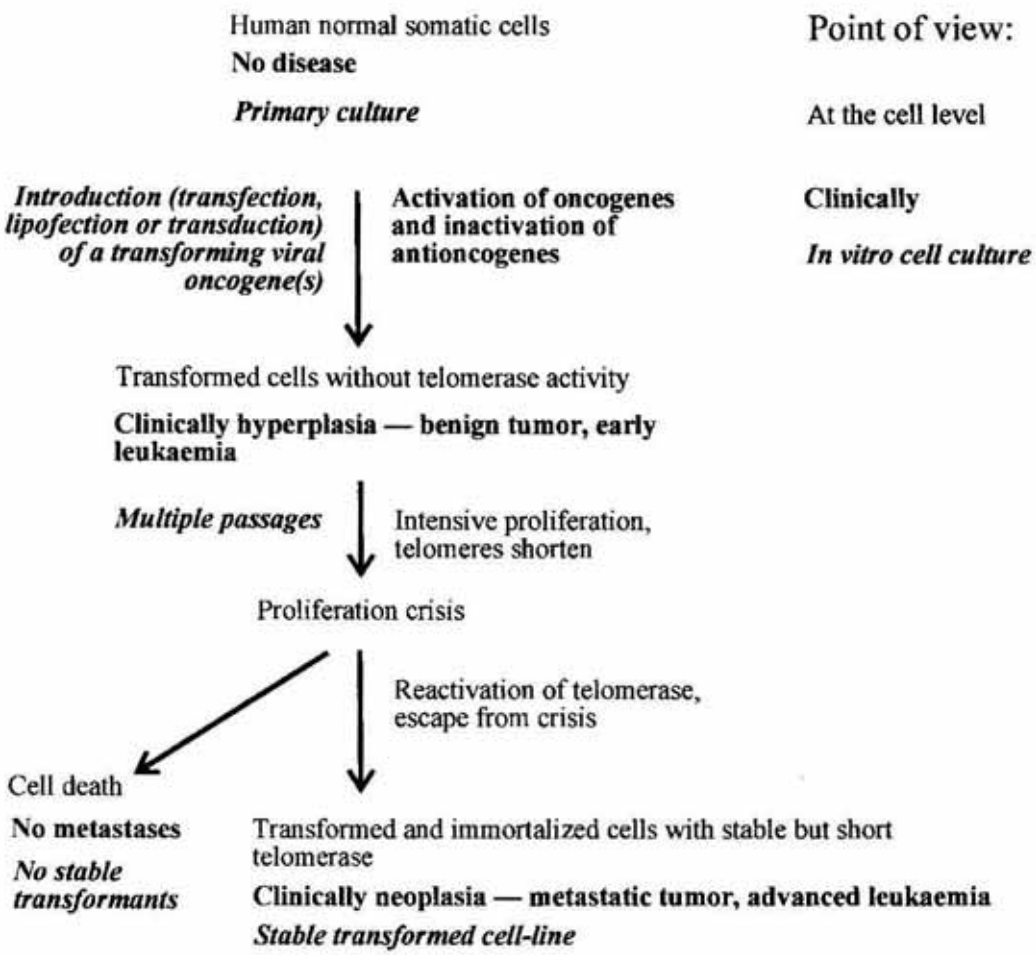

Fig. 1. New conception of carcinogenesis (in vivo and in vitro).

telomerase activity in about 85% of malignant cancers can be detected [5]. One could suppose that the new assay is too sensitive, and will detect extremely low levels of telomerase activity, too small to play a physiological function. The published results indicate that this is not true, but one cannot be sure, until the new assay is standardised and previous results are confirmed.

It was also proposed that telomerase is somehow turned off after the cell isolation. This hypothesis assumes that the phenomenon of telomerase reactivation requires unique environmental conditions, which cannot be maintained in the cell culture.

Latest data show that low telomerase activity can be also detected in bone marrow and peripheral blood leukocytes [9]. This suggests that somatic expression of the enzyme may be wider than was previously thought. It is not unlikely that telomerase activities in other somatic tissues are so low, that they cannot be detected even by the sensitive TRAP assay. Alternatively, bone marrow and peripheral blood leukocytes may be unique exceptions, and other tissues may not have telomerase at all (or more precisely nearly at all — we should not forget illegitimate transcription).

ANTI-TELOMERASE TREATMENT

Investigations focusing on the problem of telomerase activity in malignant cells are, of course, very important for understanding of the biology of cancer, but they could have an impact on therapy of malignancy, too. There are several currently ongoing clinical trials utilising strategies which are called "cancer repair" [12]. These protocols are based on intervention aimed at correcting the proliferation regulating mechanisms damaged by mutations which took place during carcinogenesis. So far, telomerase has not been usually considered to be a therapeutic target. Instead, delivering the wild-type copy of p53 antioncogene to malig-

nant cells [13, 14] (utilising the gene therapy technique) or silencing of the over-expressed oncogenes [15–17] (utilising the gene therapy, exogenous antisense oligonucleotides or conventional pharmacological inhibitors) are popular ideas. The immortalising enzyme — telomerase — is not strictly a product of oncogene, but arresting of its activity in malignant cells could cause proliferation crisis and afterwards their death.

One can argue that even if such a therapy worked, its action would be very slow, and patients would probably die before telomeres of their malignant cells would shorten to a critical length. Such a scenario is not unlikely, but there are facts which seem to be promising. Firstly, there is a negative correlation between telomerase activity and the average telomere length in transformed cells [8, 9]. This means that the telomerase activity positive transformed cells have shorter telomeres that normal cells (derived from the same tissue, and the same donor, or donor of the same age). During the early period of development of malignancy (benign tumour, early leukaemias) transformed cells divide losing telomeric DNA. When telomeres are critically short the proliferation crisis takes place. The crisis positively selects cells with reactivated telomerase. These cells are both transformed and immortalised. They survive, and can develop into malignancy. Their telomeres are stabilised, but still very short [18, 19]. An increased rate of the chromosome fusion, observed in many malignant cells, is probably the simple consequence of poor protection of chromosomes by these short telomeres. All these facts suggest that the main function of telomerase is the prevention of shortening rather than the intensive elongation of telomeres. Thus, inhibition of telomerase activity should quickly lead to reaching the proliferation limit (Hayflick's deadline).

In accordance with the latest results, telomerase activity is detectable not only in germ-line cells, but also (though it is much lower) in bone marrow and peripheral leukocytes [9]. So far, these are the only adult tissues in which telomerase has been detected. Thus, systemic anti-telomerase therapy could act as a very selective treatment for malignant tumours. Possible sterility could be probably an acceptable side-effect of therapy against advanced disease. The survival of germ-line cells with shorter telomeres would be worse, because in the next generations even faster ageing might take place. At present, it is not possible to predict potential toxic effects on leukocytes and their precursors, because both biological significance and the identity of the telomerase activity positive cells is unclear. Despite the presence of telomerase activity, telomeres in bone marrow and peripheral blood leukocytes still shorten. This was, however, shown by an assay measuring an average telomere length [9]. Thus, it could be assumed that a small subpopulation of these cells express high levels of telomerase — and do not show the telomeric decline, whereas other subpopulations express no telomerase activity — and their telomeres shorten. If so, the identity of telomerase positive subpopulation would be of great interest.

On the other hand, it is possible that all leukocytes and their precursors have some telomerase activity, but their telomeres shorten despite this activity. The rate of this shortening could be, however, slightly diminished by low level of telomerase activity. In fact, telomere loss rates vary among different cell types [8]. This could suggest that regulation of telomerase activity and telomere length is more complex than previously thought. Telomerase may be controlled not only at the level of RNA or protein expression, but also at the level of enzymatic activity. Furthermore, function of this ribonucleoprotein may not only be telomere elongation, but also maintaining the correct structure of telomeres (through incorporation into the telomeric nucleoprotein complex).

All these facts and hypothesis point to the need for great caution in the process of interpreting experimental data. Different parameters: telomere length, telomerase activity, the presence of telomerase RNA or the protein subunit, do not necessary correlate with each another. An average result will not ensure that there is no subpopulation of cells (for example stem cells) with entirely different characteristics. However, we know that most primary bone marrow stem cells (CD34+) lose their telomeric DNA during *in vitro* culturing [20]. This suggests that the telomerase activity positive cells may belong to a more differentiated compartment. This is optimistic, because toxicity against differentiated leukocyte precursors or peripheral leukocytes, even if it occurred, would not probably be life-threatening.

HOW CAN TELOMERASE BE SELECTIVELY INHIBITED?

Telomerase is a ribonucleoprotein. Its own integral RNA is used as a template for the synthesis of the DNA repeat motif characteristic of the species. Recently, a group of scientists from Cold Spring Harbour Laboratory and Geron Corporation, has cloned telomerase RNA [21]. They also proved that the introduction of a gene encoding antisense RNA targeted to telomerase RNA component into the HeLa immortalised cell-line, leads to the proliferation crisis, and cell death. Thus, antisense strategy seems to be very attractive means of telomerase inhibition.

It is hard to decide which of antisense strategies would be the best. If exogenous simple antisense strategy (deoxyoligonucleotides or their analogues) is used, the effect of inhibition will be the overall result of: 1) competition with the 3'-ended short single-stranded "overhang" at the end of telomere for binding to telomerase RNA in the holoenzyme; 2) competition with the telomerase protein component for binding to newly synthesized telomerase RNA; 3) degradation of the formed RNA:DNA dimers by RNase H. Since RNase H cannot degrade RNA:RNA dimers, mechanism 3) will not take place if we choose the gene therapy technique.

Gene therapy offers the exceptional possibility of the targeted delivery and transcription of a gene encoding antisense RNA (or other anti-telomerase genes). This could completely eliminate the potential toxicity to germ-line cells and leukocytes. Such a gene transfer would be, however, very difficult. Because the bystander-killing effect is rather unexpected (as for all "cancer repair" strategies), a 100% efficiency of transfer seems to be necessary. This would not be possible without new vector systems (e.g. a new generation of liposomes connecting high transfer efficiency of cationic liposomes with good pharmacokinetics and the targeted delivery to cancer cells of stealth liposomes [22]). When new gene transfer methods are worked out, anti-telomerase therapeutic genes could be very useful in systemic gene therapy for advanced malignancy. To enable intensive gene transfer, the targeted delivery *via* the ligand-receptor interaction should be probably excluded. The targeted transcription (use of an appropriate promoter) will be probably sufficient, since even non-targeted anti-telomerase treatment seems to be able to act very selectively against cancer cells.

Ribozyme antisense strategy (utilising exogenous or gene-derived ribozymes) is usually more effective than simple antisense strategy because ribozymes possess enzyme kinetics [23, 24]. However, there is evidence that RNA undergoing complex association with proteins may not form optimal structure for the ribozyme cleavage. For example, simple antisense strategy was more active than ribozyme strategy against RNA of a small nuclear ribonucleoprotein — U7 [25].

Antisense strategy could be targeted not only to the RNA component of the telomerase protein, but also to mRNA of the protein subunit. These two approaches are quite similar. Which of them would be more effective could depend on which factor (RNA or protein) exists in smaller quantities in the cell, and therefore limits the activity of the enzyme. Recently, it has been found that telomerase RNA is present in many human tissues which show no detectable telomerase activity [21]. This suggests that the protein component is more rigorously regulated than telomerase RNA. On the other hand, the antisense strategy targeted to the RNA component could not only prevent formation of the holoenzyme, but also inhibit the activity of created telomerase molecules. This inhibition might be very effective, since antisense oligonucleotide would compete with only 92 telomeres per cell.

The construction of any antisense molecule targeted to mRNA of the protein telomerase component requires cloning of the gene encoding this protein, which has not been done so far.

There are also other theoretically possible methods requiring the gene transfer; examples are: intracellular single-chain antibodies against the telomerase protein; transdominant mutants of the telomerase protein which can bind template, and form ribonucleoprotein without telomerase activity; "RNA decoys" similar to RNA template in that they can bind telomerase protein, but the created dimer will be inactive. These three strategies are modifications of the procedures known from gene therapy for the HIV infection, when a therapy target is to neu-

tralize some elements of the virus [26, 27]. However, they are highly experimental, and there is little experience with their use.

It is also very likely that an effective system of telomerase inhibition could be worked out in the field of "conventional" pharmacotherapy. The goal is to find selective inhibitors of the enzyme. They could be analogues of deoxynucleotides, with high affinity to telomerase, but low to DNA dependent DNA polymerases, working as terminating agents (they might stop elongation by lack of ability to create a bond with the next deoxynucleotide). The application of non-deoxynucleotide selective inhibitors is also possible. Looking for inhibitors of telomerase should include screening and drug designing. This would strongly resemble searching for inhibitors of HIV reverse transcriptase. After all, telomerase is a kind of reverse transcriptase with the built-in template.

The well known HIV reverse transcriptase inhibitor — AZT, has already been examined for its effect on the *Tetrahymena* telomerase. It was found that AZT induces fast telomere shortening in vegetatively growing *Tetrahymena* [28].

RODENT MODEL

We have a great many information about the telomerase regulation in mice tissues [29, 30]. These findings are amazing. In fact, most mouse tissues possess telomerase activity. The levels of this activity can differ a lot. Consequently, telomere length is similar in all tissues of a newborn mouse, but different in tissues of an adult mouse. In primary cell cultures of mouse fibroblasts telomerase activity is undetectable, and telomere length decreases with cell divisions. In contrast, after a frequent spontaneous immortalization, remarkable telomerase activity is present and telomeres maintain a stable length.

It was proposed that to protect them against malignancy, long-lived species need more stringent control over cell proliferation than short-lived species. This is not, however, self-evident. One could argue that long-lived species need more cell doublings — thus, should have active telomerase.

In order to explain this complicated issue other species should be investigated as well as it should be determined whether the observed telomerase activity is due to telomerase expression in all cells, or only in a limited subset of cells.

Due to the permanent telomerase expression in many somatic tissues, rodents — the most frequently used model in cancer research, apparently does not seem to be useful in this particular situation. However, some mouse tissues (for example skin) do not possess telomerase activity. Thus, an interesting proposal to use the mouse skin multistage chemical carcinogenesis model for such investigations, has been recently put forward [31].

CONCLUSIONS

The main purpose of this review is to attract attention to telomerase as a potential therapeutic target. Of course, the anti-cancer effect of inhibiting telomerase is highly theoretical. All the same, even if this assumption is false, studying the results of neutralizing telomerase should give us new knowledge about the cancer biology, ageing and the phenomenon of immortalization. Furthermore, telomerase might serve as the nearly universal malignancy marker in the cancer diagnostics. Finally, anti-telomerase treatment can be directed against fungi and protozoa. These eukaryotic unicellular organisms are (in the biological meaning) immortal and they constantly express telomerases [32–34]. Their telomeres and telomerases differ from human ones. Therefore, theoretically methods for selective inhibition of their telomerase activities could be found.

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