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BREAKTHROUGHS AND VIEWS

Telomere Crisis, the Driving Force in Cancer Cell Evolution

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Cancer cells show characteristic telomere dynamics. Their chromosomes usually have short telomeres and a high telomerase activity. The "telomere crisis model" proposed here suggests that these unique telomeric features are responsible for the progression of cancer.

Recently, the ends of chromosomes, called the telomeres, have attracted much attention because of their potential role as diagnostic or therapeutic targets for malignancies (1). As the conventional DNA replication mechanism exerted during the S phase does not synthesize the very end of the chromosome, the telomeric DNA is shortened every time the cells divide (2-5). Cells have a mechanism to counteract this progressive shortening. An enzyme called telomerase adds telomeric DNA to the 3′-end of the telomeric DNA (6, 7). The chromosomes present in the germ line cells replicate, form sperms and oocytes and are transmitted to the next generation. The length of the telomeric DNA is maintained in the germ cells during this extended replication over the generations due to the activity of the telomerase (8). After fertilization, cells in the early embryonic stage develop into two different types of cells, namely the germ cells and the somatic cells. The telomerase is down-regulated in somatic cells by an unknown mechanism (9). As a result, the length of the telomere in the somatic cells becomes shorter as the total number of cell divisions increases (10). Telomere length has been referred to as a mitotic clock, counting down from the fully wound position in germ cells to the "time-up" point in the somatic cells of elderly people (11). As cancer cells proliferate more extensively than their normal counterpart tissues, cancer cells have a reduced telomere length (12, 13). In order to continue further cell growth, the cancer cells reactivate telomerase activity to balance telomere length (14, 15). This brief description of the telomere and telomerase in cancer cells is a summary of what has been reported to date describing the implications of telomere biology in cancer development. However, recent studies, both in humans and other species, suggest that the situation is not as simple as has been thought. In this review, I will summarize these studies and propose a model, the "telomere crisis" model, to explain the clonal evolution mechanism of cancer cells from the standpoint of telomere biology.

Abbreviations used: CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; AMMoL, acute myelomonocytic leukemia; MDS, myelodysplastic syndrome.

TELOMERE LENGTH AND TELOMERASE ACTIVITY IN CANCERS

To describe the telomere dynamics of cancer cells in relation to the mechanism of cancer development, it is important to consider both the telomere length and telomerase activity. When a disease that shows a multistep progression from a pre-malignant stage to the full-blown stage is being analyzed, these properties should be described with details of when and to what magnitude they occur and how they change during the clinical course of progression. One goal of this kind of analysis is to construct a model explaining how the change in telomere function contributes to the establishment or progression of cancer cells. To achieve this goal, it is necessary to obtain accurate and quantitative measurements of telomere length and telomerase activity. Unfortunately, however, current quantitative methods are not satisfactory.

The standard method of measuring telomere length is the Southern blotting analysis of genomic DNA using telomeric TTAGGG-probes. The results obtained by this analysis represent the total length of telomeric DNAs and a proportion of subtelomeric sequences that

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do not contain the restriction site used in the study. As there is a great amount of polymorphism in the subtelomeric sequence among populations (10, 12, 16), the obtained results cannot be directly compared with different samples derived from different individuals. Another drawback is that the results may vary significantly by contamination of the samples by normal cells. In the case of solid tumors, non-cancerous cells such as inflammatory cells and fibroblasts often account for a large percentage of the cancer tissues. In such cases, the heterogeneity of the cell population prevents an accurate determination of the telomere length of the cancer cells. The TRAP assay has been used almost exclusively for the analysis of telomerase activity (1). However, this method sacrifices quantitativity for simplicity of procedure (17). Accordingly, it is not possible to examine when or by what degree the telomerase is activated.

In brief, the results obtained by these methods indicate that cancer cells have shorter telomeres and an activated telomerase (1). However, the telomere length was not shortened in all cases examined, especially in solid tumors. These irregular results may have been due to the artifactual effects described above. Another possibility is that the lengthened telomere may have originated as a requirement for cancer development, which I will discuss later. The lack of quantitativity and the possible false-negative results produced by "Taq polymerase inhibitors" in samples has hampered interpretation of the results obtained by the TRAP assay. We recently described a new PCR-based method for the determination of telomerase activity (17). This protocol, named the "stretch PCR assay", was shown to be highly quantitative and sensitive. Inclusion of a control experiment using serially diluted standard samples made it possible to quantify the activity expressed as a percentage of standard activity. A single experiment can determine the activity in a range greater than 10^5-fold. The telomere dynamics of CML and AML have been examined using Southern blotting to determine telomere length and the stretch PCR experiments to determine telomerase activity (17). The total number of cases examined in this study was relatively small. Therefore, future studies are required to confirm the results, but the synopsis is as follows. Fifteen cases of CML (7 in the chronic phase, 7 in the blastic crisis and 1 accelerated case) and 14 cases of AML (10 fresh cases and 4 relapsed cases) were examined. The telomere length was reduced in all cases compared to normal blood cells derived from similar age groups. The telomerase activity showed a number of interesting features. All cases in CML chronic phase showed very low levels of activity, similar to that found in normal blood cells. In sharp contrast, all blastic crisis cases showed strikingly high levels of activity. Therefore, it was shown that telomerase is highly activated during the progression from chronic phase to crisis. Most AML cases had some degree of telomerase activity. However, the fresh cases showed relatively low levels of activity compared to the relapsed cases. Notably, some fresh cases were telomerase negative. These results indicate two important points. First, telomerase is not essential for the establishment of cancer cells. Second, the relative titer of telomerase seems to be related to the clinical stage of leukemia, i.e. chronic phase v.s. crisis in CML and fresh v.s. relapse in AML.

OTHER FACTORS CONTROLLING TELOMERE FUNCTION

Telomeres are essential for the stable maintenance of chromosomes (18, 19). However, this function is not carried out solely by the telomerase. Another group of important regulators for telomere function is the telomere binding proteins (20). Telomere binding proteins were originally described as proteins which bound specifically to telomeric DNA, for example, to the TTAGGG-repeats in humans. The RAP1 protein in budding yeasts and the TRF1 protein in mammalian cells are examples of this class of proteins (21-24). However, another type of telomere associated proteins is now known to play an important role in telomere function. These proteins interact with the telomere DNA-binding proteins by protein/protein interactions and comprise a large functional chromosome domain, called the "telosome" (25). Examples are the SIR3/SIR4 proteins and the RIF1 protein in *Saccharomyces cerevisiae* (26, 27). These proteins associate with the RAP1 telomere DNA-binding protein and function to establish the telomere silencing effect and regulate telomere length, respectively (28, 29). Since the identification of these direct or indirect telomere-associated proteins, a picture has emerged indicating that some of these proteins negatively regulate telomere length, probably by inhibiting telomerase activity (30, 31). Therefore, telomere length and function are not determined simply by the balance between the total number of cell divisions and telomerase activity. The molecular dissection of the telomere-associated proteins has just started and further studies are needed to determine their possible roles in cancer development.

THE M1/M2 HYPOTHESIS EXPLAINS TELOMERE DYNAMICS DURING IMMORTALIZATION IN CELL CULTURE MODELS

Studies using cell culture models indicated that cells need to overcome two critical stages, Mortality stage 1 (M1) and 2 (M2) in order to become immortal (11, 32-34). M1 is a checkpoint, where shortening of the telomeres results in the arrest of growth, thus preventing further telomere loss. The M1 arrest is bypassed by

the inactivation of the p53 and Rb proteins, leading to extended cell proliferation (35). Once cells have passed through M1, they face the M2 stage, where some chromosomes lose telomere function and become extremely unstable. Some murine cells and rare cases of human cells overcome this M2 stage and grow immortally in cultures. It was found that telomerase was activated concomitantly with the M2 bypass (14). These observations of immortalized cells *in vitro* lead to the proposal that cancer cells *in vivo* activate telomerase for immortal growth (1). However, this "telomerase for immortality" hypothesis does not explain two important features of cancer cells. First, immortalization is not a unique characteristic of cancers, germ cells are also immortal. Second, this theory does not explain why most cancer cells *in vivo* have reduced telomeres, in spite of an active telomerase. It is obvious that a model which explains the characteristics of cancers *in vivo*, which I will discuss below, needs to be constructed from the standpoint of telomere dynamics.

CLONAL EVOLUTION OF CANCER CELLS

The discovery of the Ph^1 chromosome (36) and proof of the clonal origin of cancer cells (37) led to a model explaining how cancer cells progress, namely the "clonal evolution theory" (38-41). This theory suggests that all cancer cells that develop within a patient are derived from a single cell. During the reproduction of a huge number of offspring from the original cell, mutations are introduced at random. Most mutations handicap the cell's ability to grow. However, in some cases, "right" mutations which permit further efficient growth may occur. Once a cell acquires such a "right" mutation, its offspring will grow more rapidly than other cells that do not have this mutation. During the several generations of growth that follow, the mutated offspring come to dominate the cancer population. In time, another "right" mutation would be acquired by a cell and the offspring of that cell take over the following generation. This course of cancer cell development is typical of Darwinian evolution. I would like to point out two features of this hypothesis. First, "right" mutations depend on the relationship between the cancer cells and the surrounding environment (42). At the initial stage of progression, the number of cancer cells is relatively small and nutrition is sufficient. Under such conditions, the most rapidly growing cells would have an advantage and would dominate the population. However, after vigorous growth, the cancer cells become crowded and the nutritional conditions deteriorate. At this stage, cells resistant to poor conditions or cells able to metastatize have an advantage over cells simply growing. Also the occurrence of conditions for a "right" mutation to appear is a stochastic phenomenon, and not part of a definite program.

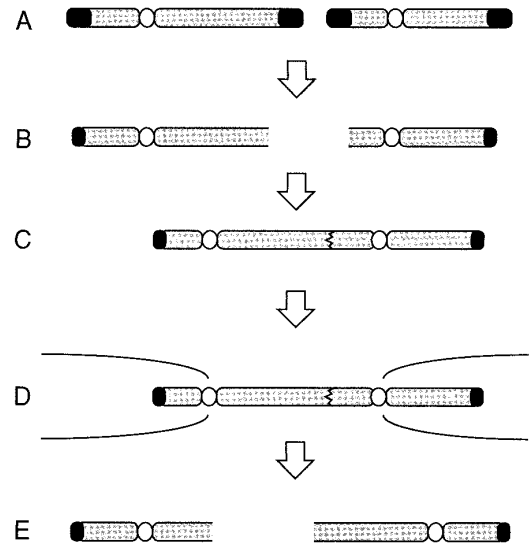

FIG. 1. Breakage-fusion-bridge cycle. Chromosomes have a telomere at each end which has a number of essential roles in chromosome maintenance (A). One role is the prevention of the end-to-end fusion between chromosomes. When two chromosomes lose telomeric function (B), these two chromosomes fuse together to form a dicentric chromosome (C). When the dicentric chromosome is segregated towards the two daughter cells during M phase, the two spindles originating from each of the two daughter cells may attach to each of the two centromeres, respectively. In this case, a single dicentric chromosome is pulled apart by the two spindles (D). As a result, a double strand break occurs between the two centromeres (E). Thus, the newly formed ends of the chromosome are non-telomeric and another cycle of breakage-bridge-fusion cycle follows.

A MODEL FOR TELOMERE DYNAMICS AND PROGRESSION OF CANCER CELLS: TELOMERE CRISIS

The results we obtained with CML and AML suggested that telomere dynamics may be of great significance in cancer development. All cases we examined showed reduced telomere length compared to their normal counterparts. The simplest explanation is that the total number of accumulated cell divisions was greater in cancer cells than in normal cells. The results indicating that there was no telomerase activity in CML chronic phase and that activity was relatively low in the initial stage of AML make it likely that cells show accelerated growth without telomerase activity during the initial stage of cancer development. Therefore, at this stage, cancer cells progressively lose telomeres. Some cells may undergo extensive proliferation until the telomere sequence is completely lost. Chromosomes without functional telomeres are very unstable. This is best illustrated by a hypothetical model, the "breakage-fusion-bridge cycle" (Fig. 1). This model was first developed by McClintock in 1941 to explain her detailed observation of maize chromosomes having newly formed ends (43). When two chromosomes lose telomeric function at their ends, these two chromosomes

fused with each other. This fused chromosome is known as dicentric chromosome, because it possesses two centromeres, derived from each of the original two chromosomes. By careful observation of the behavior of dicentric chromosomes in maize, McClintock found that they were highly unstable. During mitosis, the two centromeres on a single chromosome are pulled apart by spindles derived from the two daughter cells. As a result, physical tension builds up in the region between the two centromeres and a new breakage occurs within this region. The resultant two chromosomal fragments do not have a functional telomere at the newly formed ends, which leads to another cycle of the fusion, bridge and breakage cycle. A variety of aberrant chromosomes are produced by this mechanism (44) (Fig. 1). However, as long as the telomerase is not activated, no healing of the newly formed chromosome ends occurs. Thus, cells undergoing the breakage-fusion-bridge cycle in these stages eventually die. This model explains why CML chronic phase does not show progression and is essentially self-limited.

However, during the course of the disease, some cancer cells happen to acquire telomerase activity as a "right" mutation, as in the blastic crisis in CML. The offspring of this cell have an advantage over other cells for the following reasons: At this late stage of cancer, many limitations are imposed on cancer cells, such as limited nutrition, space and the iatrogenic administration of anti-cancer therapeutics. In order to proliferate efficiently, cancer cells need to deal with these obstacles by acquiring the appropriate "right" mutations for each limitation. As these "right" mutations are only produced by chance from among a large number of random mutations, cancer cells must be genetically unstable in order to produce a series of "right" mutations efficiently. As I stated, the simple breakage-fusion-bridge cycle is not unstable but catastrophic, because cells without end-healing activity will eventually die. However, if cells have a reduced telomere (leading to "breakage-fusion-bridge cycle") and an active telomerase (healing of shuffled aberrant chromosomes), they can actively produce stable and aberrant chromosomes. Under such conditions, the chance to form a "correctly" modified chromosome (such as deletion of anti-oncogene) will be significantly raised. This ability to randomly modify chromosomes may give cancer cells an enormous advantage, because these cells can respond rapidly to changes in the environment by chromosomal evolution. I propose that the conditions discussed here, namely a reduced telomere and an active telomerase, form a powerful driving force for cancer cells to carry out clonal evolution. I suggest that this particular condition in telomere dynamics be called "telomere crisis". Under clinical conditions where these criteria are met, such as CML crisis and AML relapse cases, the rate of progression of the disease is greatly accelerated, which leads to a poor prognosis. It has been reported that cancer patients with high levels of telomerase activity showed poorer prognosis compared to those with lower levels (45). This was explained by the fact that telomerase is more active in vigorously proliferating cancer cells than in resting cells (34). I would argue that another important factor determining the prognosis of the telomerase-positive patients is the high level of clonal evolution, which would result in a high probability of the cancer becoming resistant to therapeutic agents.

The occurrence of breakage-fusion-bridge cycle has been shown in rodent cells, where it functions as a mechanism of amplification for drug-resistance genes (46-48). However, it should be emphasized that so far no direct evidence has been obtained to show that clinical human cancers progress by a mechanism of telomere crisis, as I have proposed here. One major reason for this failure may be that the dicentric chromosomes postulated to occur in the breakage-fusion-bridge cycle are too unstable to replicate clonally. Thus, it is likely that the key intermediate, the dicentric chromosome, has escaped detection by conventional cytogenetics. Recently, a highly sensitive PCR technique has been developed for the detection of these unstable chromosomes (Takahashi et al., unpublished). In future, efforts should be made to demonstrate that telomere crisis is an actual molecular event.

Indirect support for the telomere crisis model has been provided by a rare type of chromosomal anomaly, namely jumping translocation. About 20 leukemia cases with this abnormal karyotype have been reported (49). The anomaly comprises a region of chromosome apparently jumping to the tips of many other chromosomes. Recently, we have experienced jumping chromosomes in a case of AMMoL which had progressed from MDS (Hatakeyama et al., unpublished). We have demonstrated for the first time that the jumping is actually towards the shortened telomeres and does not involve a minute translocation. This fusion is best explained by the assumption that the preceding MDS had resulted in excessive trimming of telomere sequences. The resultant telomere dysfunction may have led to a special type of fusion, that is, jumping.

The telomere crisis model explains several characteristic features of clinical cancers. For a cell to obtain a "right" mutation, two highly stochastic phenomena are necessary. One is the production of a "right" mutation by random chromosomal changes. The second is the successful healing of the broken ends by telomerase. As the probability of these two events occurring is considered to be very low, it would take a long period of time for them to occur simultaneously to confer the "right" mutation. The stochastic nature of molecular evolution may explain why clinical progression of disease is not continuous but occurs in an unpredictable fashion in many cases.

Telomere length is generally reduced in most cases

of leukemia (50, 51). In contrast, it is common for telomeres to be lengthened, instead of shortened, in solid tumors (52). Two reasons may be envisioned for these apparent contradictions to the telomere crisis model. First, as described earlier, determination of telomere length by Southern blotting analysis is susceptible to misinterpretation due to artifacts. An unexpectedly large amount of normal cells contaminating a tumor sample may be responsible for the some of the irregular results. The second possible reason is related to the difference in the clonal evolution mechanisms in leukemia and solid tumors. Most hematological malignancies do not form tumors. The cells proliferate and are circulated as unicellular cells. Therefore, each leukemic cell competes directly for more efficient proliferation. As a result, the offspring of a cell having obtained a "right" mutation will dominate the population in a short period. In contrast, in a typical solid tumor, the cancer cells settle in one place without further movement. Therefore, competition among the offspring having different mutations is limited to close neighbors. In this case, it would take more time for a "right" clone to dominate the entire population. Under these conditions, most successful clones will have greater genetic stability than the leukemias, because a particular phenotype suited to a particular environment must be maintained for a relatively long period of time for a clone to dominate the population. This reasoning may explain why some solid tumors have long telomeres. Cell lines are interesting extremes. Human cell lines derived from cancers often have very high telomerase activity and long telomeres. As these cells have been maintained for a long time under defined growth conditions, they may have evolved to a plateau. In this case, all actual chromosomal changes may result in less efficient growth. Therefore, cell clones having long telomeres and being genetically stable have the "best" phenotype and are thus selected. Finally, the properties specific to leukemias makes hematological malignancies a useful model for the molecular dissection of cancer development.

Finally, it is evident that the telomere crisis model does not explain the entire evolutional mechanism of cancer cells, as it only accounts for the development mechanism of certain chromosomal changes. It is important to develop a model that will explain the other types of genetic and epi-genetic changes found in cancers, such as point mutations.

Many efforts are currently under way to develop potential telomerase inhibitors for pharmaceutical use (53). These agents are anticipated to form a new class of anti-cancer drugs. It is expected that cancer cells will die after treatment with telomerase inhibitors, because the telomere length of the cancer cells will become so short that the chromosomes cannot be maintained. Some opponents argue that it will take such a long time for the cancer cells to lose telomere function that this type of treatment is unrealistic. I would like to propose another possible anti-cancer mechanism of the potential telomerase inhibitors. According to the telomere crisis hypothesis, both a reduced telomere and active telomerase are necessary for the clonal evolution of chromosomes. If a drug inhibits the telomerase action, the telomere crisis mechanism no longer works and the driving force for clonal evolution will be abrogated. This will result in a reduction in the rate of progression of these diseases and a significant improvement in the patients' prognosis. Molecular identification of the genes encoding the protein components of human telomerase (Nakayama et al., submitted) will stimulate the development of this new therapeutic tool.

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