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Telomerase Activity and Metastasis: Expansion of Cells Having Higher Telomerase Activity within Culture Lines and Tumor Tissues

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Tumor cells with metastatic potential may have a high telomerase activity that augments telomeric DNA repeats, allowing the cells to escape from the inhibition of cell proliferation due to shortened telomeres. We examined the expression level of telomerase activity using the telomeric repeat amplification protocol among a series of cell lines obtained by repeated transplantation of a mouse fibrosarcoma. The lines could be grouped into three; one has no metastatic potential, and the other two show metastatic abilities after intravenous or subcutaneous injection. Comparison of their telomerase activity indicated that more malignant lines had higher activity. A similar relation was seen in metastatic nodules formed through clonal expansion from the heterogeneous population of inoculated cells; clonality was monitored in terms of variable patterns of subtelomeric repeats. The results suggest that a high level of telomerase activity may not be requisite for metastasis, but may confer a propensity to dominate in a tumor tissue.

Key words: Telomere — Telomerase activity — Metastasis

Human and mouse telomeres consist of hundreds to thousands of tandem repeats of six-base consensus sequence of TTAGGG, with associated proteins.[1–4] The telomeric repeats are attached to the nuclear matrix near the nuclear membrane and protect the end of a chromosome from fusion and recombination. Size reduction of telomeric repeat arrays is observed during the aging of normal cells and in many human carcinomas, and is ascribed to the loss of repeats during cell divisions.[5,6] Somatic cells stop dividing when the telomeric ends of at least some chromosomes have been shortened to a critical length.[2–4,7] Telomerase is an RNA-dependent DNA polymerase that synthesizes and adds telomeric repeats onto the ends of chromosomes.[8,9] This process is supposed to take place in germline and stem cells to compensate for the loss of telomeres. Surveys of telomerase activity in a wide variety of human cancerous and noncancerous tissues have detected the activity in almost all advanced tumors and some adult tissues containing germline and stem cells, but not in most somatic cells.[10–20] This suggests a tight linkage between telomerase activity and cell immortality; i.e., synthesis of DNA at chromosome ends by telomerase may be necessary for indefinite proliferation.

The expression level of telomerase activity required for persistent tumor growth and the mechanism of its regulation are not clear, however. In human tumor samples, the activity level seems to vary in tumor tissues, and telomere lengths differ.[10,19] Some tumors show little telomerase activity, whereas others exhibit strong activity even after 1000-fold dilution of the extracts. This variability may be ascribed to inappropriate sampling, because consistent upregulation of telomerase activity was observed in experimentally induced mouse tumors compared to normal and hyperplastic tissues.[21–23] Generally, high levels of telomerase activity have been observed in advanced cancers including metastatic lesions and in tumors with altered telomere length.[4,10–23] This suggests that tumor cells with malignant properties are inclined to possess or possibly have acquired higher telomerase activity. In order to examine whether this is the case, we examined the telomerase activity in a series of mouse fibrosarcoma cell lines with different metastatic abilities, as well as in freshly developed mouse tumors and metastatic nodules. Such analysis using an experimental animal system may provide a clue to linking telomerase activity to metastasis. Here we describe a tendency of increase in telomerase activity during malignant progression of tumor cells, which suggests that a high level of telomerase activity may confer a propensity to dominate in a tumor tissue.

MATERIALS AND METHODS

Cell lines and cells MST tumor cell lines were established as previously described.[24] Four cell lines, Y1, Y2, Y1-1 and Y1-2, were newly established. Y1 is the parental line of Y1-1 and Y1-2. A new name is given to each of the other lines used here to show better the relationships; the changes are as follows: 2HC to P4-1, 1L1 to P4-2, 2L8 to P4-3, SCL1 to P4-31, Lb to P4-32, 3K4 to P4-33. C23 and Y2 originate from MST1-4 cells, and P4 is a

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Table I. Telomerase Activity Assayed with TRAP

	Cell lines[a]	Activity[b]	[c]
	HeLa cell	1.00	1.00
A	MST1-4	0.08	0.06
	C23	0.10	0.09
	Y2	0.08	0.07
	Y1	0.16	0.10
B	P4	0.13	0.10
	Y1-1	0.17	0.11
	P4-1	0.19	0.20
	P4-2	0.18	0.12
C	P4-3	0.28	0.25
	Y1-11	0.18	0.17
	P4-31	0.43	0.36
	P4-32	0.41	0.32
	P4-33	0.70	0.77
D	MST-S1	0.88	0.62
	S1-1-T	0.57	0.57
	S1-1-M	0.80	0.61
	S1-2-T	0.22	0.19
	S1-2-M1	0.47	0.39
	S1-2-M2	0.30	0.36
	S1-3-T	0.17	0.22
	S1-3-M	0.47	0.88

a) Cell lines classified into group A are originally derived from a tumor and do not develop metastatic lesions after intravenous injection of 5×10^5 cells. On the other hand, group B and group C lines have metastatic potentials after intravenous and subcutaneous injection, respectively. MST-S1 cells were derived from an MST tumor. T and M in the end of a cell line name indicate cells from a tumor and a metastatic nodule, respectively.
b) Activity is expressed as a ratio obtained by calculation of each TRAP signal divided by the signal of HeLa cells. Five micrograms of protein of extracts was used.
c) One microgram of protein of extracts was used.

descendant of C23. The other P lines are derived from P4. Their properties of tumor and metastatic nodule formation are summarized in Table I. The cells were cultured at 37°C in 5% CO_2 and 95% air in α-modified minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin.

Tumor formation and metastasis Eight- to twelve-week-old F_1 mice between C57BL/6(B6) females and MSM males were used to obtain tumors and metastatic lesions by subcutaneous injection of MST cell lines (5×10^5 cells). MSM mice are an inbred strain derived from Japanese wild mouse, *Mus musculus molossinus*. Mice were kept in plastic cages in an air-conditioned room.

Telomerase assay Preparation of cell extracts and assay of telomerase activity were carried out according to Kim *et al.*[10] The telomeric repeat amplification protocol (TRAP) assay was done using a fluorescence-labeled primer.[25] In brief, cells were washed once in phosphate-buffered saline, and resuspended at 10^6 cells per 20 μl of ice-cold lysis buffer containing 10 m*M* Tris-HCl (pH 7.5), 1 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 m*M* phenylmethylsulfonyl fluoride, 5 m*M* β-mercaptoethanol, 0.5 CHAPS (Sigma Chemical Co., St. Louis, MO), and 10% glycerol. The suspension was incubated on ice for 30 min and centrifuged at 100,000*g* at 4°C for 30 min. The supernatant was removed, quick-frozen on dry ice, and stored at −80°C. The concentration of protein was measured by use of the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL). An aliquot of extract containing 5 μg or 1 μg of protein was used for each TRAP assay. Extension of oligonucleotide TS by telomerase was performed at 23°C and the reaction mixture was subjected to 27 or 32 polymerase chain reaction (PCR) cycles after heat treatment. The PCR products were electrophoresed on a 10% polyacrylamide gel using a Pharmacia ALF DNA sequencer. To estimate telomerase activity in samples, we compared the intensity of TRAP assay-generated DNA ladders using analysis software (Pharmacia DNA Fragment Manager V 1.1).

Pulsed-field gel electrophoresis (PFGE) of DNA High-molecular-weight DNA was prepared from cultured cells in agarose plugs as previously described.[26,27] The agarose plugs were digested with restriction enzymes under the manufacturer's recommended conditions, and subjected to electrophoresis using a Beckman Gene Line apparatus. Electrophoresis was run in 1×TAFE buffer at 200 V for 36 h with a 3-s to 9-s switching interval. The gel was directly hybridized to [γ-^{32}P]ATP-labeled $(TTAGGG)_4$ oligonucleotide in 5×SSPE (1×SSPE=10 m*M* sodium phosphate [pH 7.0], 0.18 *M* NaCl, 1 m*M* EDTA) containing 0.1% SDS and 10 μg/ml sonicated denatured salmon sperm DNA at 37°C for 16 h.[26,28] The gel was washed and exposed to X-ray film (Fuji, Tokyo) for 18 to 36 h with an intensifying screen at −80°C.

RESULTS

The TRAP assay is a highly sensitive PCR-based method for detecting telomerase activity, and produces ladder signals in a gel.[10] With this assay, we examined thirteen cell lines obtained by repeated subcutaneous transplantation of the original MST1-4 cells.[24] The lines were classified into three groups with respect to metastatic potential. MST1-4, C23 and Y2 form a group with no metastatic ability. The second group, including Y1, P4, Y1-1, P4-1 and P4-2, shows metastatic ability after intravenous injection, but not after subcutaneous injection. The third group (P4-3, Y1-11, P4-31, P4-32 and P4-33) had acquired a more malignant potential, i.e., metastatic ability after subcutaneous injection. Each of the

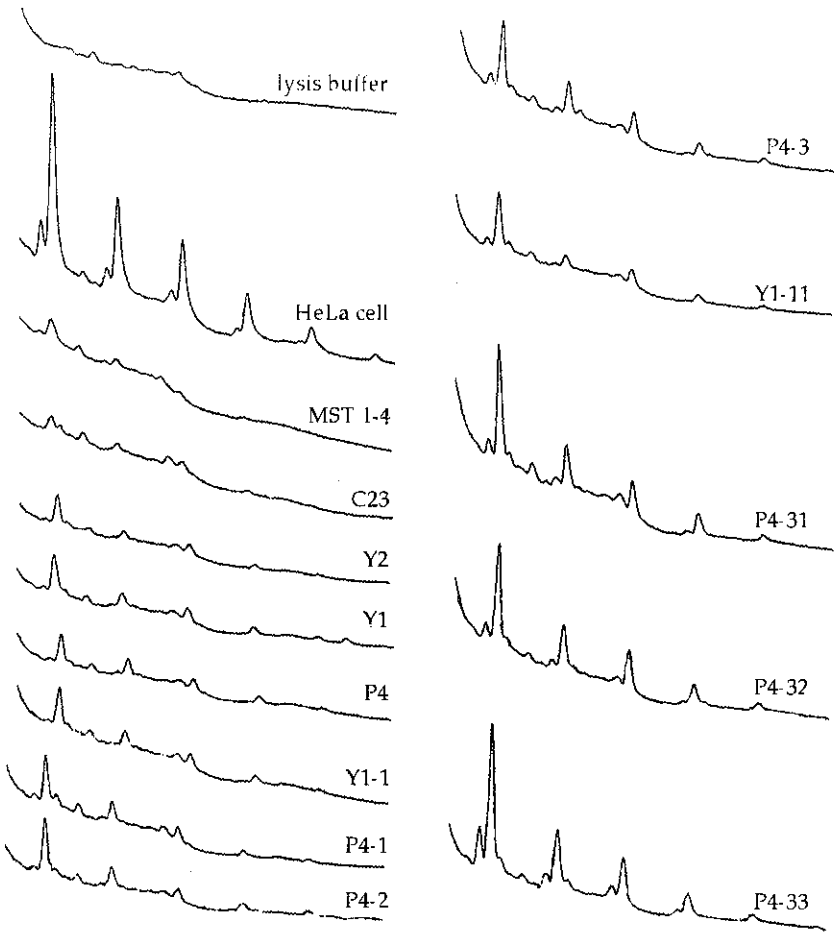

Fig. 1. Ladder signals showing telomerase activity. Multiple peaks were obtained by electrophoresis of PCR products in TRAP assay. Each peak corresponds to a different copy number of telomeric repeats synthesized by telomerase.

samples gave ladder signals on a sequence gel (Fig. 1). Quantification of the ladder signals (representing the telomerase activity) revealed clear differences among the samples. To verify the differences, we carried out assays under the following two conditions. (1) We examined samples at two different concentrations of extract to minimize variation in the TRAP assay. (2) All samples assayed were prepared from cells harvested from the fourth to eighth day after starting culture of frozen stocks (see "Discussion"). Table I, A–C shows telomerase activities of the thirteen lines. The five lines belonging to the second group each gave a similar activity and the activity was higher than that of the first group, the cell lines without metastatic potential. The third-group lines tended to show still higher activities than the other two groups. These results suggest that enhancement of metastatic capability parallels the increase of telomerase activity.

The mouse cell line, MST-S1, had been maintained for more than 6 months and hence probably comprised a heterogeneous cell population. Injection of these cells afforded three tumors and four metastatic nodules. TRAP assay of their extracts showed that all the samples had telomerase activity, and Table ID gives their activities (ladder patterns not shown). The activities in the four metastatic nodules (S1-1-M, S1-2-M1, S1-2-M2, and S1-3-M) were all higher than those in the respective original tumor samples (S1-1-T, S1-2-T, and S1-3-T), although the tumors exhibited lower activities than that of MST-S1 cells.

PFGE analysis was carried out for MST-S1 and the derivatives to examine changes in the lengths of telomeric repeat restriction fragments. The pattern consisted of a smear and bands (Fig. 2). The smear was composed of telomeric repeats flanked by unique sequences ranging from 5 kb to 40 kb in length, and the bands comprised telomeric repeats and adjacent minor satellite arrays of longer sizes. All or most mouse chromosomes are known to have a minor satellite close to the centromeric end and telomeric repeats flanked by a unique sequence at the opposite end.[27,29–31] The pattern of one tumor sample, S1-1-T, was similar to that of MST-S1, but the patterns of the other two tumors (S1-2-T and S1-3-T) were different.

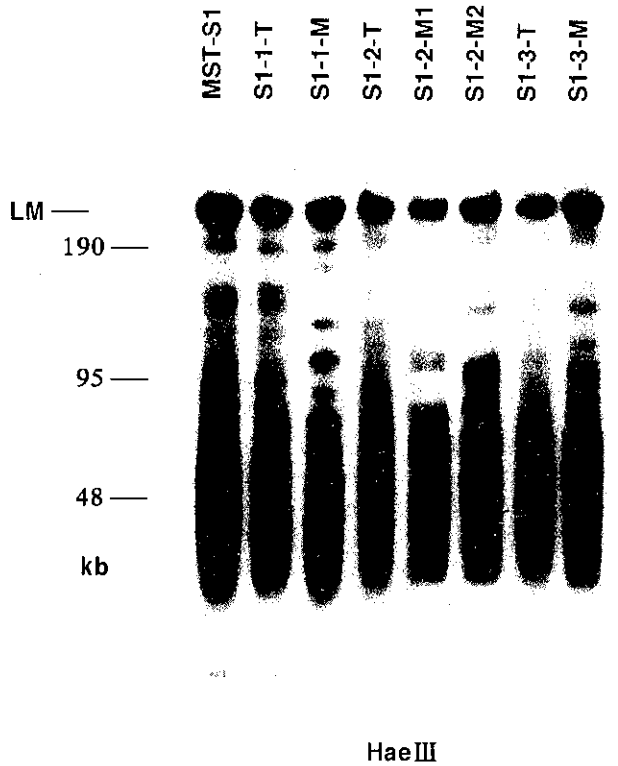

Fig. 2. PFGE analysis of telomeric repeat restriction fragments. *Hae* III digests were separated by PFGE and hybridized to the telomere probe of $(TTAGGG)_4$. Positions of size markers are indicated on the left. LM shows the position of the upper limit of separation.

The four samples from metastatic nodules also gave distinct band patterns. Note that two samples from metastatic nodules (S1-1-M and S1-2-M1) showed shorter smears than those of their parental tumors, but the other two (S1-2-M2 and S1-3-M) did not show much difference (see "Discussion"). The observed differences in subtelomeric repeats of tumor and metastatic cells probably reflected heterogeneity of the MST-S1 cells, i.e., the different cell lines resulted from clonal growth and subsequent selection of certain clones during tumor formation and metastasis.[32-34]

DISCUSSION

In the present study, we have examined the telomerase activity in a variety of mouse fibrosarcoma lines with different metastatic potentials. The more malignant tumor cells with respect to metastasis appear to have higher telomerase activity (Table I, A–C). This suggests that cells with higher telomerase activity come to dominate in a cell population during repeated processes of *in vitro* culture, and artificial and spontaneous metastases. This tendency is also observed in tumors and metastatic nodules that were developed by injection of MST-S1 cells. Cells from metastatic lesions exhibited telomerase activities higher than those of their parental tumors (Table ID). The differences in telomerase activity are not due to an artifact in the TRAP assay or to change of the activity during *in vitro* culture. Eight samples were obtained from MST-S1 at one-day intervals after starting culture from frozen stocks and subjected to TRAP assay. They showed similar activities by TRAP assay from the fourth to eighth day (data not shown). Accordingly, all samples assayed were harvested within this period. Such persistence has already been reported in human cancer cell lines.[10, 17, 18, 35]

Telomerase regulation takes place at multiple levels of tumor development and progression.[21] One is at the level of cell proliferation; telomerase activity is upregulated upon mitotic stimulation of mouse splenic T cells[23] and during *in vitro* culture of primary mouse cells.[36] Additional levels of regulation or cell-type-specific regulatory pathways must exist, because there are a number of studies showing less clear correlations between telomere activity and cell proliferation.[10, 11, 21, 37] In yeast, *Saccharomyces cerevisiae*, it is known that a large number of genes affect telomere regulation.[38] The finding that telomerase activity appeared to increase during metastasis of MST tumor cells suggests that the activity is also regulated in the metastatic process. However, it is difficult to understand how telomerase activity affects metastasis. One possible explanation is that low telomerase activity, leading to shortened telomeres, would be a disadvantage in terms of the persistent proliferation required for tumor metastasis. As a result, cells having higher telomerase activity could give rise to metastatic lesions through selection. However, mice have extraordinarily long telomeres of more than 20 kb in somatic tissues, which should allow for considerable expansion of malignant cell populations without activation of telomere maintenance functions.[29, 30] The mouse telomeres do not appear to decay at a faster rate.[21, 29, 30] Besides, the long telomeres do not confer upon mouse cells a corresponding increase in life span. A second possibility is that there might be some short telomeres. Indeed, cell-cycle arrest often occurs at an early point when the telomeres detected by PFGE analysis are not yet critically shortened.[4] Such putative sensor telomeres could induce an activation signal and may require activated telomerase for their maintenance. A third possibility was proposed, i.e., that the activated tumor telomerase serves to repair damaged chromosomes.[21]

Telomeres are thought to anchor chromosomes by interactions with the nuclear envelope.[3, 4] Cells with shortened telomeres and altered telomere sequences ex-

hibit aberrant nuclear morphology, cell division and chromosome loss in yeast, probably because such cells have topologically unstable chromosomes in the nucleus.[39,40] It has been suggested that shortened telomeres in cancers provide a signal to reactivate telomerase.[4,10,35,41] In accordance with this, it was reported that altered length of telomere repeats was frequently associated with high telomerase activity.[10,11,19] Therefore, the shortening of telomeres observed in some of the metastatic nodules of MST-S1 (Fig. 2) might have generated a signal to increase telomerase activity. Recently a human telomere-repeat binding protein, TRF1, has been isolated which negatively regulates telomere elongation.[42] Overexpression of TRF1 results in gradual telomere shortening, whereas introduction of a dominant-negative mutant protein of TRF1 provides marked telomere elongation. Proteins having similar function have also been isolated in other species.[43–45] Reduction of repeat-tract binding sites for TRF1 or related proteins would be a signal that leads to an increase of telomerase action. The mechanisms of reactivation and maintenance of telomerase activity in cancer cells remain to be further investigated.

Telomerase consists of protein and RNA components and hence the telomerase activity is influenced by the amount of RNA. The amount of RNA in cells was examined during mouse tumor development and progression.[21,22] The level of RNA is upregulated in the early preneoplastic stages and further increases during progression, whereas telomerase activity is only detected in late-stage tumors. Intriguingly, it is evident that RNA level does not parallel the level of telomerase activity. Therefore, although we did not investigate the RNA, it seems unlikely that telomerase RNA is the major determinant of the telomerase activity in MST cells showing different metastatic potentials.

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