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Advances in Brief

Cytological Detection of Telomerase Activity Using an *in Situ* Telomeric Repeat Amplification Protocol Assay[1]

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Abstract

A previously reported highly sensitive assay for measuring telomerase activity on cell and tissue extracts indicates that most human tumor tissues, but not cells adjacent to tumors, have detectable telomerase activity. Although this assay has provided a significant amount of information about the presence or absence of telomerase activity, it does not indicate whether all cells within a tumor have telomerase activity or whether only a subset does. The present report demonstrates the ability to advance this technology to an *in situ* assay. Using fluorescent telomerase primers and *in situ* PCR, we show that telomerase activity can be detected at the cellular level. This study demonstrates that telomerase activity is not detected in normal cells but is detected in tumor cells of clinical specimens and in tumor-derived cell lines.

Introduction

The ribonucleoprotein, telomerase, is thought to be important in maintaining the stability of telomeres (the ends of linear chromosomes) by compensating for the TTAGGG repeat loss that occurs in its absence (1, 2). The enzyme is active in embryonic cells and in adult male germ-line cells (3) but is undetectable in normal somatic cells except for proliferative cells of renewal tissues, *e.g.*, activated lymphocytes (4–6), basal cells of the epidermis (7), and intestinal crypt cells (8). Somatic cells that do not have telomerase activity eventually stop dividing and become senescent when their telomeres have eroded to a critical length (9, 10). Thus, it has been proposed that the synthesis of DNA at the chromosome ends by telomerase may be required to sustain the indefinite proliferation of most malignant tumors (11, 12).

Although human telomerase RNA has been cloned (13), the protein(s) that associate with it have not yet been identified. Most studies to date have measured telomerase activity in heterogeneous tissue extracts. With the introduction of the sensitive TRAP[3] (14, 15), telomerase has been reported to be detectable in small tissue samples from almost all tumors and tumor-derived cell lines (16–24). Using this assay and an internal standard to semiquantitate telomerase activity (25), it has been demonstrated that some tumors have very high activity, which often correlates with poor outcome, whereas other tumors have low telomerase activity, which in some instances correlates with a good prognosis (20, 26). It is not known if all cells within a tumor have telomerase activity or if only a subset does. An *in situ* assay to detect telomerase activity levels in cytological samples has not been reported.

We reported previously the use of a fluorescent-labeled TRAP assay to detect telomerase activity semiquantitatively (27–29). Using fluorescent TS and CX telomerase primers and an automated laser fluorescence DNA sequencer, we demonstrated that the RNase-sensitive six-base periodic peaks corresponded to the ladder of the original TRAP assay (14, 15), indicating that FITC-labeled primers can be used to detect telomerase activity.

The present report describes an *in situ* assay for telomerase activity that gives negative results for cells lacking telomerase activity (normal cells) and cell lines that have been experimentally immortalized via a telomerase-independent mechanism, but gives a positive fluorescent signal in tumor cells derived from clinical specimens and tumor-derived cell lines expressing telomerase activity.

Materials and Methods

Samples and Cell Lines. Peripheral blood from four normal volunteers and two leukemia and lymphoma patients was obtained after obtaining informed consent. Peripheral blood mononuclear cells were separated by using Ficoll-Hypaque centrifugation. The telomerase activity in mononuclear cells from healthy volunteers (normal resting lymphocytes) was compared to that following 72 h exposure to PHA (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions (PHA-stimulated lymphocytes). We used TIG-1 cells (a telomerase-negative immortal fibroblast cell line) as a negative control. We analyzed nine leukemia cell lines (HEL, K562, TS9;22, SS9;22, MOLM-1, U937, OM9;22, HAL-01, and HL60) and six solid tumor-derived cell lines (COLO #320DM, COLO #691, COLO #694, COLO #699, COLO #711N, and PANC-1).

***In Situ* PCR.** Cells were washed in cold medium or phosphate buffer saline (PBS), cytospinned (400 rpm for 3 min) onto nonfluorescent silane-coated slide glasses, and air dried quickly. Adherent cells were trypsinized, washed in cold medium, and then cytospinned. Twenty-five μl containing 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 μM deoxynucleoside triphosphates, 1 μg of T4 gene 32 protein (Boehringer Mannheim, Indianapolis, IN), BSA (0.1 mg/ml), 2 units of Taq DNA polymerase, and 10 pmol of FITC-labeled (5′-end labeling using FluorePrime; Pharmacia Biotech, Uppsala, Sweden) TS forward-primer (5′-AATCCGTC-GAGCAGAGTT-3′), according to the original and fluorescent TRAP method (14, 28, 29), were placed within each frame, and the slides were incubated 30 min at 22°C in a dark box. After TS extension, 25 μl of the same solution but with 10 pmol of FITC-labeled (5′-end labeling) CX reverse-primer (5′-CCCT-TACCCTTACCCTTACCCTTA-3′) were added, coverslips were sealed, heated to 90°C for 1.5 min to inactivate telomerase, and then amplified using a Hybrid OmniSlide System thermocycler (National Labnet Co., Woodbridge, NJ). The PCR conditions were 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. Slides were washed in tap water and then sealed with a coverglass using MacIlvaine buffer/glycerin solution (1:1 v/v). Cells were observed using a fluorescence microscope using B-filter (Nikon, Tokyo, Japan).

Mixture Experiment. TIG-1 cells were cultured for 48 h with 0.737-μm latex spheres, which are taken up by phagocytosis (30). Latex bead-labeled

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[3] The abbreviations used are: TRAP, telomeric repeat amplification protocol; PHA, phytohemagglutinin.

TIG cells were trypsinized, mixed with K562 cells, cytospinned on silane-coated nonfluorescent slides, and processed for *in situ* PCR.

Results

Detection of Telomerase Activity in Stimulated Lymphocytes. We first tested the *in situ* PCR assay to detect telomerase activity in PHA-stimulated lymphocytes. It has been shown previously that normal resting lymphocytes essentially lack telomerase activity, whereas PHA-stimulated lymphocytes contain detectable levels of telomerase activity, using the original TRAP assay (4–6). Similar results were obtained with the *in situ* PCR assay (Fig. 1). Normal resting lymphocytes had very weak fluorescence (Fig. 1*A*), whereas PHA-stimulated lymphocytes gave a bright fluorescence signal (Fig. 1*B*). Because the primers themselves are fluorescent, the very weak fluorescence in normal resting lymphocytes may be nonspecific; thus, we concluded that they are essentially negative. This signal in PHA-stimulated lymphocytes was brighter when both TS and CX primers were FITC labeled (Fig. 1*B*) than if only one of the primers contained FITC (Fig. 1, *C* and *D*). When lymphocytes are stimulated to divide and subjected to PCR without including Taq polymerase, there is not as strong a positive signal as observed when Taq polymerase is included (data not shown). Because telomerase is a very low abundant enzyme complex, without PCR we cannot distinguish real telomerase from the background of the fluorescent primers. This indicates that the fluorescent primers subjected to PCR do not by themselves contribute to the fluorescence observed. On the basis of the results of this experiment, we used both TS and CX primers labeled by FITC for further studies. No fluorescent signals were detected when nonlabeled TS and CX primers were used, indicating that PCR by itself does not produce a fluorescence signal (Fig. 1*E*). Almost no fluorescent signal was detected if FITC-labeled TS and FITC-labeled CX primers were applied without performing PCR (Fig. 1*F*), confirming that the positive signals were not due to nonspecific sticking of the labeled primers.

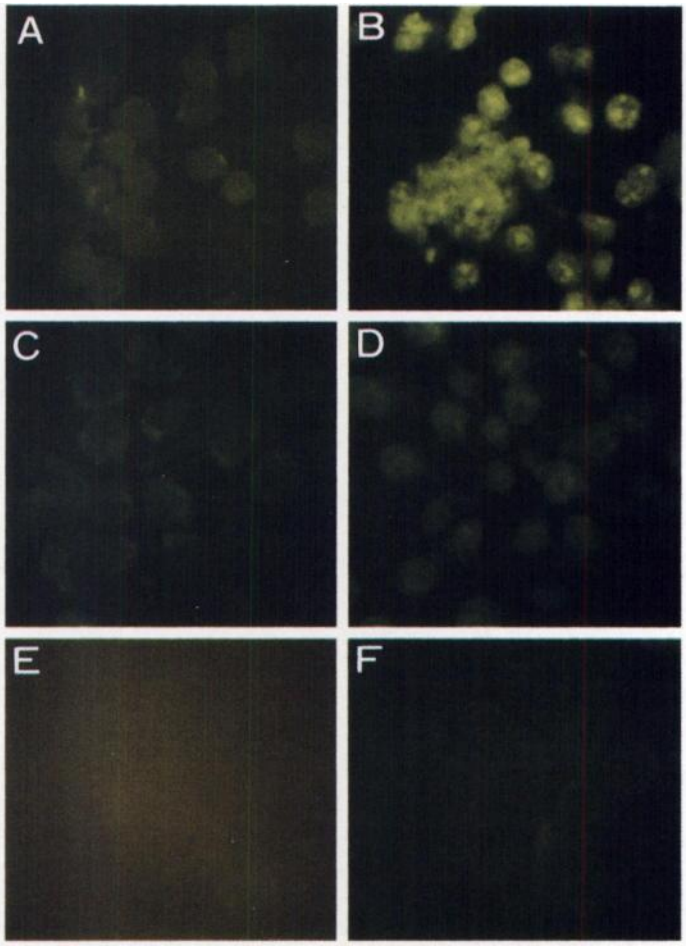

Fig. 1. In normal resting lymphocytes, only very weak fluorescence was detectable (*A*), whereas PHA-stimulated (72 h) normal lymphocytes obtained from a healthy volunteer had a bright fluorescence signal (*B*). The fluorescent signal in PHA-stimulated lymphocytes was brighter when both primers were fluorescent (*B*) than if only one fluorescent primer was used: FITC-labeled TS primer and nonlabeled CX primer (*C*) or nonlabeled TS primer and FITC-labeled CX primer (*D*). When nonlabeled TS and CX primers were used, no fluorescent signals were detected (*E*). Only very weak fluorescent signals were detectable if both fluorescent primers were used, but the PCR amplification step was eliminated (*F*).

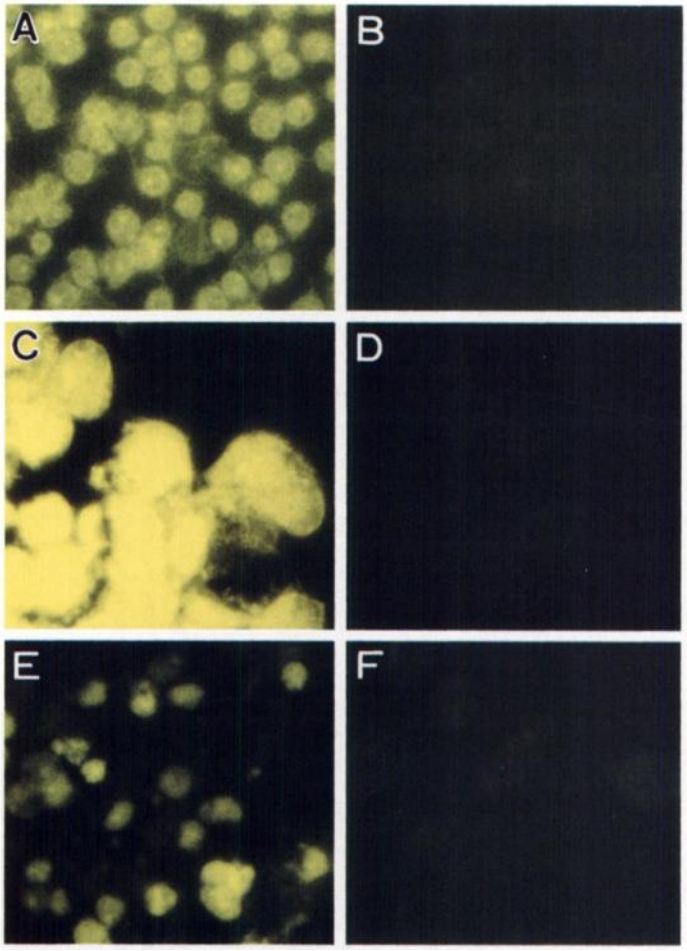

Fig. 2. Fluorescent signals corresponding to telomerase activity in acute myeloid leukemia cells with (*A*) or without (*B*) PCR cycle. Leukemia cells had bright fluorescence corresponding to telomerase activity after performing PCR. Lung cancer cells obtained from pleural fluid demonstrated bright fluorescence corresponding to telomerase activity (*C*), but only very weak fluorescent signals were seen in lung cancer cells without PCR (*D*). Established tumor-derived cells, COLO #320DM (colon carcinoma cell line; *E*) had bright fluorescent nuclei. In contrast, the telomerase activity-negative fibroblast cell line, TIG-1, did not show bright fluorescence (*F*).

***In Situ* PCR Using Leukemia Cells and Tumor-derived Cell Lines.** We performed *in situ* PCR using peripheral blood mononuclear cells obtained from a patient with acute myeloid leukemia. Bright fluorescent signals were detectable after performing PCR (Fig. 2*A*), whereas only weak fluorescent signals were observed without PCR (Fig. 2*B*). In cells obtained from a patient with lymphoma, bright fluorescent signals were also observed (data not shown). Lung cancer cells obtained from pleural fluid demonstrated bright fluorescence, indicating that *in situ* PCR (Fig. 2*C*) could detect telomerase activity using clinically obtained cytological materials, but only weak fluorescence was detected without PCR (Fig. 2*D*). The bright fluorescence after PCR was again demonstrated in the established solid tumor cell

line (Fig. 2*E*). In contrast, TIG-1, a telomerase-negative cell line, had only very weak background levels of fluorescence (Fig. 2*F*).

The ability to specifically identify telomerase-positive cells in a heterogeneous population was confirmed in mixing experiments. Telomerase-negative TIG-1 cells were first labeled by allowing them to phagocytize latex spheres (30), which were then washed and mixed with telomerase-positive K562 cells. Examination of the cells after the *in situ* PCR telomerase assay showed that latex bead-labeled (TIG-1) cells exhibited only background fluorescence, whereas many of the unlabeled cells (which are a mixture of K562 cells and those TIG-1 cells that failed to phagocytize beads) fluoresced brightly (Fig. 3). This demonstrates that telomerase-negative cells do not pick up fluorescent products from adjacent positive cells, and that the bright fluorescence of telomerase-positive cells is not a technical artifact due to factors such as different photographic exposures or selection of areas with high backgrounds.

We also examined the telomerase staining pattern in a variety of cultured human tumor-derived cell lines (Fig. 4*A*; Table 1). The *in situ* TRAP assay showed two relatively distinct types of fluorescent staining: bright *versus* dim nuclear fluorescence, both of which appeared punctate, accompanied by a much weaker cytoplasmic staining. The incidence of bright fluorescence in nuclei ranged from 14 to 67% in various human tumor-derived cell lines, all of which contained telomerase activity detected by the original or fluorescent TRAP assay (Table 1). Within each cell type, the fractional distribution of bright fluorescence was consistent over multiple assays. These observations indicate that the fluorescent signals obtained using the *in situ* PCR assay are likely to represent telomerase activity at the cellular level (Fig. 4). No PCR (Fig. 4*B*), RNase treatment (10,000 units/ml, 37°C, 30 min; Fig. 4*C*) or heat treatment (70°C, 15 min; Fig. 4*D*) resulted in loss of the bright fluorescence signals, suggesting that the bright fluorescent signal in the nuclear portion of each cell depended on the presence of a ribonucleoprotein.

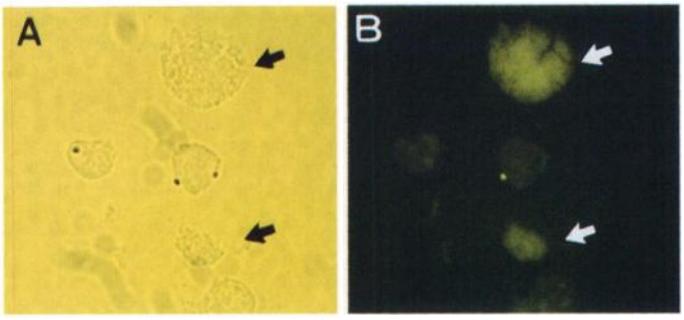

Fig. 3. A mixture of latex sphere-labeled telomerase-negative cells (TIG-1) and unlabeled telomerase-positive cells (K562) demonstrated that only nonlabeled K562 cells (*arrows*) had bright fluorescent signals (*A*, visible light; *B*, fluorescent light).

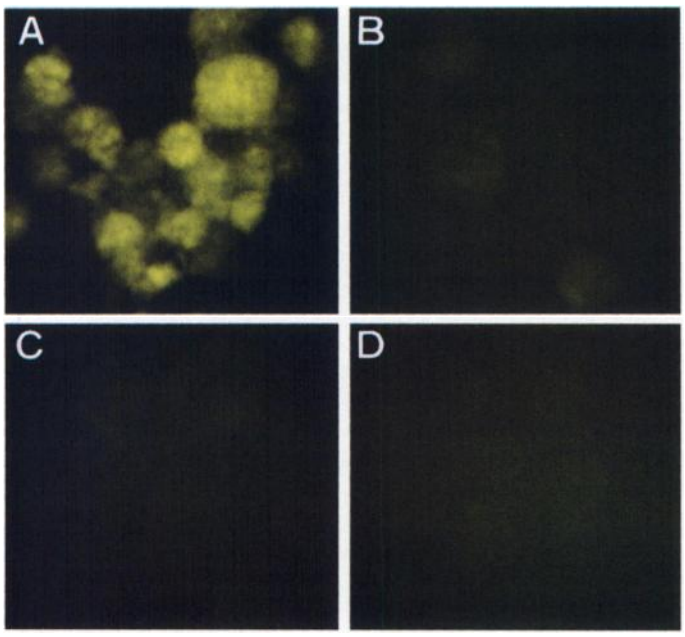

Fig. 4. Fluorescent signals in the leukemia cell line K562 with (*A*) or without (*B*) PCR. No bright fluorescent signals were detected after RNase treatment (10,000 units, 37°C; *C*), or heat treatment (70°C, 15 min; *D*).

Table 1 *Detection of telomerase activity using an* in situ *telomerase PCR assay in various human tumor-derived cell lines*

In each cell line, 100 cells were examined using a fluorescent microscope, and the data are represented as a percentage. Data are expressed as means ± SD obtained from three independent experiments.

	Fluorescent signals at:		
	Nucleus (%)[a]	Cytoplasm	Telomerase activity
Human leukemia cell lines			
HEL	17.0 ± 3.6	Yes	144
K562	53.7 ± 2.1	Yes	106
TS9;22	63.3 ± 4.2	Yes	70
SS9;22	43.0 ± 9.0	Yes	68
MOLM-1	30.0 ± 4.6	Yes	9.4
U937	30.3 ± 4.2	Yes	174
OM9;22	48.7 ± 10.6	Yes	150
HAL-01	35.7 ± 4.5	Yes	190
HL60	14.0 ± 4.3	Yes	72
Colon carcinoma			
COLO #320DM	67.0 ± 3.6	Yes	48
Breast cancer			
COLO #691	22.3 ± 7.2	Yes	9.0
Uterine carcinoma			
COLO #694	34.3 ± 9.7	Yes	254
Lung adenocarcinoma			
COLO #699	58.0 ± 3.5	Yes	100
Melanoma			
COLO #711-N	27.3 ± 3.5	Yes	13.2
Epitheloid carcinoma			
PANC-1	37.0 ± 4.4	Yes	85.4
Normal resting lymphocytes			
Aged 26 years	0	Yes[b]	0.9
Aged 29 years	0	Yes[b]	0.8
Aged 42 years	0	No	0.4
Aged 63 years	0	No	0.2
PHA-stimulated normal lymphocytes (72 h)			
Aged 30 years	Yes[c]	Yes	8.9
TIG-1 (telomerase-negative cell line)	0	No	0

[a] Nucleus indicates bright fluorescence in the nucleus, and the remaining cells had punctate fluorescence. Telomerase activity was determined by fluorescent-TRAP assay using an internal telomerase assay standard (25). Relative telomerase values (telomerase activity) are calculated by the area of telomerase signals per area of internal telomerase assay standard defined by an automated DNA sequencer and Fragment manager system (27–29).

[b] Yes indicates weak positive signal.

[c] Yes indicates positive punctate fluorescence in the nucleus.

Discussion

The present results demonstrate that the use of fluorescent primers permits the TRAP assay to be used as an *in situ* technique for the microscopic identification of individual cells expressing telomerase activity. It is known that a variety of telomerase-competent cells can regulate the expression of telomerase, repressing its activity when they become quiescent or differentiate to a postmitotic state (31, 32). In the present study, we have confirmed that the up-regulation of telomerase that is observed when quiescent lymphocytes are activated to divide can be observed cytologically using the *in situ* TRAP assay.

Although freshly isolated acute myelogenous leukemia and lymphoma cells exhibited uniformly bright fluorescent signals in the *in situ* assay, a very heterogeneous signal was seen within each popula-

tion of 15 different established tumor-derived cell lines (Table 1). Virtually all of our current information concerning telomerase expression is based on the assay of cell or tissue extracts, which measures the average telomerase activity in a population of cells. Telomerase activity does not vary with the G_1, S, and G_2-M phases of the cell cycle (31); therefore, the heterogeneity we observed is unlikely to be cell cycle related. However, some hints that telomerase expression in clonal populations may not be uniform are beginning to emerge. For example, some freshly isolated small subclones of established cell lines are initially telomerase negative but re-express telomerase when they are expanded, suggesting that telomerase is fluctuating between on and off states.[4] Our results support this interpretation and suggest that the level of telomerase within individual cells can vary widely. Although the uniform staining we observed in the freshly isolated clinical samples suggests that heterogeneity is not a technical artifact, we cannot at this time rigorously exclude the possibility that cultured cells have altered characteristics that make uniform penetration of the assay reagents more difficult to achieve.

Our initial attempts to use the *in situ* PCR telomerase assay on frozen sections of pathological materials have been unsuccessful. It is likely that modifications to prevent solubilization/diffusion of the enzyme or to increase the permeability of the samples will be needed before the technique can be applied to tissues.

One of the goals of early cancer detection is to validate new molecular markers that could supplement or replace the older anatomically or cytologically oriented ones, such as X-ray, endoscopy, or fluid cytology. Although microscopic cytopathology is presently considered the "gold standard," there are many instances in which cytology cannot make accurate diagnoses or risk-stratify patients into those with favorable and those with unfavorable prognosis. With the knowledge that telomerase activation occurs so frequently in cancer, it is possible that it could become a useful new modality for supplementing microscopic cytopathology in the early detection of cancer cells, perhaps as a surrogate biomarker in population-based screening to detect lesions prior to the onset of tissue invasion. Because telomerase activity is also expressed in proliferative cells of renewal tissues including inflammatory cells, tissue- and cell extract-based telomerase activity assays may need to be supplemented with cytologically based methods such as the *in situ* PCR telomerase activity assay presented in this report. Our findings suggest that the *in situ* PCR assay described here is able to detect telomerase at the cellular level and should be applicable to demonstrate telomerase activity in cytological specimens, including sedimented cells in voided urine, oral rinses/sputa, colon effluents, and hematological as well as other materials obtained by fine-needle aspirates and brushes. It is hoped that the refinement of the *in situ* telomerase activity assay may in the future provide useful information in clinical oncology.

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