Cytological Detection of Telomerase Activity Using an in Situ Telomeric Repeat Amplification Protocol Assay

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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 TRAP3 (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 #75235, and PANC-1).

In Situ PCR. Cells were washed in cold medium or phosphate buffer saline (PBS), cytopsinized (400 rpm for 3 min) onto nonfluorescent silane-coated slide glasses, and air dried quickly. Adherent cells were trypanized, washed in cold medium, and then cytopsinized. Twenty-five μl containing 20 mm Tris-HCl (pH 8.3), 1.5 mm MgCl2, 63 mm KCl, 0.05% Tween 20, 1 mm EGTA, 50 μm deoxyribonucleoside 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'-AATCCGTCGAGCCAGAGTT-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'-CCCTTATCCCTACCCCTACCCCT-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 coverslip using MacElvaine 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

References

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4 The abbreviations used are: TRAP, telomeric repeat amplification protocol; PHA, phytohemagglutinin.
TIG cells were trypsinized, mixed with K562 cells, cytopspun 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. 1A), whereas PHA-stimulated lymphocytes gave a bright fluorescence signal (Fig. 1B). 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. 1B) 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. 1E). Almost no fluorescent signal was detected if FITC-labeled TS and FITC-labeled CX primers were applied without performing PCR (Fig. 1F), confirming that the positive signals were not due to nonspecific sticking of the labeled primers.

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. 2A), whereas only weak fluorescent signals were observed without PCR (Fig. 2B). 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. 2C) could detect telomerase activity using clinically obtained cytological materials, but only weak fluorescence was detected without PCR (Fig. 2D). The bright fluorescence after PCR was again demonstrated in the established solid tumor cell line.
line (Fig. 2E). In contrast, TIG-1, a telomerase-negative cell line, had only very weak background levels of fluorescence (Fig. 2F).

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 only very weak background levels of fluorescence (Fig. 2F). 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. 4A; 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. 4B), RNAse treatment (10,000 units/ml, 37°C, 30 min; Fig. 4C) or heat treatment (70°C, 15 min; Fig. 4D) 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.

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-

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

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Nucleus (%)</th>
<th>Cytoplasm</th>
<th>Telomerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEL</td>
<td>17.0 ± 3.6</td>
<td>Yes</td>
<td>144</td>
</tr>
<tr>
<td>K562</td>
<td>53.7 ± 2.1</td>
<td>Yes</td>
<td>106</td>
</tr>
<tr>
<td>TS9:22</td>
<td>63.3 ± 4.2</td>
<td>Yes</td>
<td>70</td>
</tr>
<tr>
<td>SS9:22</td>
<td>43.0 ± 9.0</td>
<td>Yes</td>
<td>68</td>
</tr>
<tr>
<td>M05:4-1</td>
<td>30.0 ± 4.6</td>
<td>Yes</td>
<td>9.4</td>
</tr>
<tr>
<td>U937</td>
<td>30.3 ± 4.2</td>
<td>Yes</td>
<td>174</td>
</tr>
<tr>
<td>OM9:22</td>
<td>48.7 ± 10.6</td>
<td>Yes</td>
<td>150</td>
</tr>
<tr>
<td>HAL-01</td>
<td>35.7 ± 4.5</td>
<td>Yes</td>
<td>190</td>
</tr>
<tr>
<td>HL60</td>
<td>14.0 ± 4.3</td>
<td>Yes</td>
<td>72</td>
</tr>
<tr>
<td>Colon carcinoma COLO #320DM</td>
<td>67.0 ± 3.6</td>
<td>Yes</td>
<td>48</td>
</tr>
<tr>
<td>Breast cancer COLO #691</td>
<td>22.3 ± 7.2</td>
<td>Yes</td>
<td>9.0</td>
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<tr>
<td>Uterine carcinoma COLO #694</td>
<td>34.3 ± 9.7</td>
<td>Yes</td>
<td>254</td>
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<tr>
<td>Lung adenocarcinoma COLO #699</td>
<td>58.0 ± 3.5</td>
<td>Yes</td>
<td>100</td>
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<td>Melanoma COLO #711-N</td>
<td>27.3 ± 3.5</td>
<td>Yes</td>
<td>13.2</td>
</tr>
<tr>
<td>Epitheloid carcinoma PANc-1</td>
<td>37.0 ± 4.4</td>
<td>Yes</td>
<td>85.4</td>
</tr>
<tr>
<td>Normal resting lymphocytes</td>
<td>0</td>
<td>Yes*a</td>
<td>0.9</td>
</tr>
<tr>
<td>Aged 26 years</td>
<td>0</td>
<td>Yes*b</td>
<td>0.8</td>
</tr>
<tr>
<td>Aged 29 years</td>
<td>0</td>
<td>Yes*b</td>
<td>0.4</td>
</tr>
<tr>
<td>Aged 42 years</td>
<td>0</td>
<td>No</td>
<td>0.2</td>
</tr>
<tr>
<td>Aged 63 years</td>
<td>0</td>
<td>No</td>
<td>0.2</td>
</tr>
<tr>
<td>PHA-stimulated normal lymphocytes (72 h) Aged 30 years</td>
<td>Yes*c</td>
<td>Yes</td>
<td>8.9</td>
</tr>
<tr>
<td>TIG-1 (telomerase-negative cell line)</td>
<td>0</td>
<td>No</td>
<td>8.9</td>
</tr>
</tbody>
</table>

a| b| c
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). Yes indicates weak positive signal.

Yes indicates positive punctate fluorescence in the nucleus.

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/ml, 37°C, 30 min; Fig. 4C), or heat treatment (70°C, 15 min; Fig. 4D).
Acknowledgments

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