Telomerase Activity in Human Endometrium

Satoru Kyo,1 Masahiro Takakura, Takafumi Kohama, and Masaki Inoue
Department of Obstetrics and Gynecology, School of Medicine, Kanazawa University, 1-1 Takaramachi, Kanazawa, Ishikawa 920, Japan

Abstract

Human uterine endometrium undergoes a complex pattern of changes in proliferation and secretory activity during the menstrual cycle. In the present study, telomerase activity in normal endometrium was examined using a non-radioisotope PCR-based telomeric repeat amplification protocol assay. Various levels of telomerase activity were detected in the 60 normal endometrial samples examined, depending on the phase of the menstrual cycle. Of 21 proliferative-phase endometrial samples, 20 (95%) expressed telomerase activity, whereas 8 of 19 (42%) secretory-phase or menstrual endometrial samples did (P = 0.002). Five of nine (56%) samples from atrophic endometrium from postmenopausal women also expressed telomerase activity. Eleven of 21 (52%) endometrial samples in the secretory phase expressed high telomerase activity detectable after 100-fold dilution of extracts, whereas none of the 19 endometrial samples from the secretory phase or during menstruation and none of the 9 postmenopausal endometrial samples did (P < 0.001). The highest activity was observed in the late proliferative phase, but activity dramatically decreased with the progression of the secretory phase. Surprisingly, the levels of telomerase activity detected in the late proliferative phase were comparable to those detected in the endometrial cancers examined. Immunohistochemical analysis of the expression of proliferating cell nuclear antigen revealed that telomerase activity is closely correlated with endometrial cell proliferative activity. These findings indicate that normal endometrium expresses telomerase, the activity of which changes dramatically over the course of the menstrual cycle, suggesting in turn that telomerase is a regulated enzyme linked to cellular proliferation and that hormone functions may be involved in its regulation.

Introduction

Telomeres are the distal ends of human chromosomes composed of tandem repeats of the sequence TTAGGG (1). Possible functions of telomeres include stabilization of chromosome ends and prevention of their degradation, end-to-end fusions, rearrangements, and chromosome loss. Human telomeres undergo progressive shortening with cell division through replication-dependent sequence loss at DNA termini (2). A possible mechanism for this shortening of human telomeres is the somatic repression of telomerase, a specialized ribonucleoprotein polymerase containing an integral RNA with a short template element that directs the synthesis of telomeric repeats at chromosome ends (3, 4). Telomerase is thought to compensate for the loss of telomeric repeats with cell divisions. Normal somatic cells express low or undetectable levels of telomerase activity, resulting in the progressive loss of telomeric sequences with cell divisions (5, 6). It has been proposed that the immortalization of cells involves a mechanism halting telomere shortening and averting the deleterious consequences of uncapped chromosome ends. The expression of telomerase thus seems to be concomitant with the attainment of immortality by tumor cells (7, 8). A variety of cell lines and malignant tumors have been found to specifically express telomerase activity (9–17), whereas normal somatic cells do not express this activity, suggesting that telomerase activation may be a critical step in cell immortalization and oncogenesis. A highly sensitive PCR-based TRAP assay has recently been established. Using this method, weak telomerase activity has also been detected in some normal somatic cells such as hematopoietic cells, epidermal keratinocytes, and cervical epithelial cells (18–22). The functional significance of this activity in normal somatic cells remains to be elucidated.

During the reproductive years, a pattern of cyclic changes in human endometrial activity is established. Human endometrium undergoes a complex pattern of proliferation, secretory activity, and breakdown over an approximately 28-day period. Proliferative activity of endometrium increases as the proliferative phase proceeds and is maximal in the late proliferative phase, which corresponds to the preovulatory peak of estrogen. In contrast, endometrial proliferative activity decreases in the secretory phase, with minimal activity in the late secretory phase and during menstruation. Thus, the proliferative activity of endometrium changes during the menstrual cycle, and it is possible that estrogen plays an essential role in the regulation of the proliferative activity of endometrial cells. This drastic change in proliferative activity in the endometrium during the menstrual cycle prompted us to examine telomerase activity in the endometrium. We examined telomerase activity in 60 normal endometrial samples as well as 17 endometrial cancers using the non-radioisotope TRAP assay. Our findings indicated that normal human endometrium expresses telomerase activity, which seemed to be correlated with the phase of the menstrual cycle and tightly connected with the proliferative activity of endometrial cells. Interestingly, the highest level of activity observed in proliferative-phase endometrium was comparable to that observed in endometrial cancer cells. These findings suggest that telomerase might be a regulated enzyme and that it may play a significant role in the maintenance of the cyclic renewal of the endometrium, and more widely, in the regulation of cellular proliferation.

Materials and Methods

Tissue Samples. Sixty normal human endometrial samples and 17 endometrial cancer tissue samples were obtained from hysterectomy specimens from women undergoing surgery performed at the School of Medicine, Kanazawa University Hospital and National Kanazawa Hospital. The normal endometrial samples included 40 samples from women with regular menstrual cycles, 9 samples from postmenopausal women, and 11 samples from women undergoing hormonal therapy. The collected endometrial tissues were sampled for histopathological diagnosis, and the remaining portions of samples were frozen at −80°C until used for the TRAP assay. The phase of the menstrual cycle was determined by histological examination of the tissues and confirmed by the day of the menstrual cycle.

TRAP Assay. Frozen samples were suspended in ice-cold wash buffer [10 mM HEPES-KOH (pH 7.5), 1.5 mM MgCl2, 10 mM KCl, and 1 mM DTT], pelleted, and homogenized in 200 μl of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-pro-
panesulfonate (Sigma), and 10% glycerol. After 30 min of incubation on ice, the lysate was centrifuged at 15,000 × g for 30 min at 4°C, and the supernatant was frozen and stored at −80°C. The protein concentration in the extract was measured by Bradford assay. Five g of protein were used for each TRAP assay.

For estimation of telomerase activity, the extracts were diluted 10- and 100-fold. For RNase treatment, 5 g of protein were preincubated with 1 g of RNase A for 30 min at 37°C. Assay tubes were prepared by sequencing 0.2 g of CX primer (5'-CTTACCCCTACCTCCCACTT-3') under a wax barrier (Ampliwax; Perkin-Elmer Corp., Foster City, CA). Each extract was assayed in 50 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 1.5 mM MgCl2, 60 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 µM deoxynucleotide triphosphates, 0.2 g of TS primer (5'-AATCCGTCGAGCAGAGT-3'), 1 g of T4g32 protein (Boehringer Mannheim), and 2.5 units of Taq DNA polymerase (Wako, Osaka, Japan). Each reaction mixture contained an internal telomerase assay standard for quantitative estimation of the levels of telomerase activity and the identification of false-negative tumor samples containing Taq polymerase inhibitors (13). After a 30-min incubation at 23°C for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90°C for 3 min and then subjected to 31 PCR cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 60 s. The PCR products were electrophoresed on a 12% polyacrylamide gel and visualized by SYBR Green I nucleic acid gel stain (FMC Bioproducts, Rockland, ME).

Immunohistochemistry. The expression of PCNA in endometrium was determined immunohistochemically by the avidin-biotin-peroxidase method using 10% formalin-fixed and paraffin-embedded sections. Briefly, the tissue sections were deparaffinized, hydrated in graded ethanol, and immersed for 20 min in 0.3% hydrogen peroxide to block endogenous peroxidase activity. After washing with PBS, they were treated with 10% normal horse serum for 20 min to inhibit nonspecific binding to antisera. Monoclonal antibody against PCNA (DAKO, Kyoto, Japan) at a 100-fold dilution was applied to the sections at 4°C. After rinsing in PBS, the sections were incubated for 30 min with biotin-labeled anti-mouse IgG (Vector, Burlingame, CA). They were then treated with the avidin-biotin complex (PK-4000; Vector) at room temperature. Negative controls included sections incubated with normal horse serum instead of the primary specific antibody. Sites of peroxidase activity were visualized with 3,3'-diaminobenzidine substrate kit (SK-4100; Vector). Ten endometrial glands were randomly selected for estimation of PCNA activity, which was determined as the mean ratio of PCNA-positive cells/gland. PCNA activities were divided into two groups, low (positive cells more than 5% but less than 30%) and high (more than 30% positive cells).

Statistical Analysis. Statistical analysis was performed using a χ2 test to evaluate the significance of the differences. P < 0.05 was considered to be statistically significant.

Results

Telomerase Activity in Endometrium during a Menstrual Cycle. We used a non-RI TRAP assay for the detection of telomerase activity. In the non-RI TRAP assay, the amplified PCR products of telomeric repeats were visualized with a highly sensitive DNA staining reagent, SYBR Green (FMC BioProducts). The sensitivity of the assay was examined in our recent study (17) and found to be as high as that of the conventional RI-TRAP assay.

A total of 40 endometrial samples during a menstrual cycle were examined including 21 samples in the proliferative phase, 16 samples in the secretory phase, and 3 samples obtained during menstruation. TRAP assay using 5-µg extracts revealed that 29 of 40 cases (73%) exhibited positive signals for telomerase activity (Table 1). Twenty of 21 endometrial samples (95%) in the proliferative phase were telomerase-positive, whereas 9 of 19 samples (47%) in the secretory phase or obtained during menstruation were telomerase-positive (P = 0.002). Because the signal intensities varied among samples, we next sought to estimate telomerase activity by dilution TRAP assay using 10-fold (0.5 µg)- and 100-fold (0.05 µg)-diluted extracts (Fig. 1). Telomerase activity detected after 100-fold dilution was classified as high, whereas that detected after 10-fold dilution but not after 100-fold dilution was classified as moderate. Activity that disappeared in diluted extracts was classified as low. Eleven of 21 (52%) endometrial samples in the proliferative phase expressed high levels of telomerase activity, and 7 exhibited moderate activity. None of the 3 endometrial samples in the early proliferative phase, 2 of 6 samples in the mid-proliferative phase, and 9 of 12 samples in the late proliferative phase expressed high activity. In contrast, none of 16 endometrial samples in the secretory phase or obtained during menstruation expressed high activity (P < 0.001, versus proliferative phase endometrium), and only 3 samples exhibited moderate activity. Two of 4 endometrial samples in the early secretory phase exhibited moderate activity, whereas only 1 of 12 samples in the late secretory phase did. None of 3 endometrial samples obtained during menstruation exhibited high or moderate activity. These results are summarized in Fig. 2 and suggest that normal human endometrium expresses telomerase activity, the level of which varies depending upon the phase of the menstrual cycle. Activity increased with progression of the proliferative phase, and the highest activity was observed in the late proliferative phase, whereas activity decreased with progression of the secretory phase, and the lowest activity was observed in late secretory phase and during menstruation.

Telomerase Activity in Postmenopausal or Hormone-treated Endometria. Telomerase activity was examined in atrophic endometrial samples from postmenopausal women (Table 1). Five of nine (56%) were found to be telomerase-positive. One of these five exhibited moderate activity, whereas the other four exhibited low activity. None of the five had high activity. Eleven endometrial samples from

<table>
<thead>
<tr>
<th>State of endometrium</th>
<th>Negative</th>
<th>Low&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Moderate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>High&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative phase</td>
<td>(n = 21)</td>
<td>1</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Early</td>
<td>(n = 3)</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Mid</td>
<td>(n = 6)</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Late</td>
<td>(n = 12)</td>
<td>0</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Secretory phase</td>
<td>(n = 16)</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Early</td>
<td>(n = 4)</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Late</td>
<td>(n = 12)</td>
<td>7</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Menstruation</td>
<td>(n = 3)</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>(n = 3)</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Drug administration</td>
<td>(n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen</td>
<td>(n = 2)</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>(n = 2)</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Estrogen + progesterone</td>
<td>(n = 4)</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gn-RH analogue</td>
<td>(n = 3)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>(n = 17)</td>
<td>2</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Positive using 5 µg of extract, but negative using 0.5 µg of extract.
<sup>b</sup> Positive using 5 µg or 0.5 µg of extract, but negative using 0.05 µg of extract.
<sup>c</sup> Positive using 5 µg, 0.5 µg, or 0.05 µg of extract.
Correlation between Telomerase Activity and Proliferative Activity of Endometrium. The present results revealed phase-dependent telomerase expression in endometrium during the menstrual cycle. We therefore examined the correlation between telomerase activity and proliferative activity of endometrial cells. Proliferative activity of endometrial cells was evaluated immunohistochemically by testing for the expression of PCNA. A total of 42 endometrial samples from throughout the menstrual cycle were examined. As expected, endometrial samples in the mid- or late proliferative phase were likely to exhibit high PCNA activity in the nuclei of glandular cells, whereas those in the late secretory phase or obtained during menstruation exhibited weak activity (Fig. 3). The correlation between PCNA activity and telomerase activity in each endometrial sample was examined, and the results are shown in Table 2. Sixteen of 19 (84%) PCNA-positive samples exhibited moderate or high telomerase activity, whereas 4 of 23 (17%) PCNA-negative samples did ($P < 0.001$). High telomerase activity was only observed in PCNA-positive cases. Six of seven cases expressing high telomerase activity also had high PCNA activity. Thus, a significant correlation was found between telomerase activity and PCNA activity in endometrial cells. However, four PCNA-negative cases expressed moderate telomerase activity. These four cases all exhibited PCNA expression in stromal cells instead (Fig. 3D). Thus, it is possible that stromal cells also contribute to changes in telomerase expression during the menstrual cycle. Taken together, these findings suggest that telomerase activity in endometrium is strongly correlated with the proliferative activity of endometrial cells.

Discussion

In the present study, we found that normal endometrial cells expressed telomerase, the activity of which was regulated during the menstrual cycle. Telomerase activity in endometrium seemed to increase with progression of the proliferative phase, and the highest activity was observed in the late proliferative phase. The level of
activity in this phase was comparable to that in endometrial cancer. In contrast, telomerase activity dramatically decreased during the secretory phase, and no activity or faint activity was detected in the late secretory phase and during menstruation. Atrophic endometrium in postmenopausal women also exhibited activity, but only at low levels. This characteristic pattern of changes in telomerase expression during the menstrual cycle was correlated with that in the proliferative activity of endometrial cells.

Until recently, due to low sensitivity of the conventional telomerase assay, telomerase had been thought to exist only in immortal cells and germ-line cells, and not in normal somatic cells. However, recent studies using a highly sensitive TRAP assay have demonstrated that some types of normal somatic cells express low levels of telomerase activity, indicating that human telomerase is not restricted to immortal cells and that the somatic expression of this enzyme may be more widespread than had previously been inferred from the shortening of human telomeres. In particular, physiologically regenerating normal somatic cells such as hematopoietic cells and basal cells of epidermal or cervical keratinocytes are likely to express telomerase, suggesting the possible involvement of telomerase activity in maintaining the regenerative potential of the tissue (18–22). Our observations support this concept and also demonstrate that normal somatic cells can express high levels of telomerase activity comparable to those of cancer cells. Because significant telomerase activation has previously been considered specific for cancer cells, the detection of telomerase activity has been thought to be useful for diagnosing cancers, including uterine endometrial cancer (17). The present results, however, suggest that attention should be given to the possibility of false-positive signals from normal cells, particularly in the case of endometrial samples.

Initial studies have demonstrated that telomerase activation is a critical step in cellular immortalization and oncogenesis (7, 9). Subsequent studies have also suggested the presence of a correlation between telomerase activation and the malignant potential of tumors. In some types of cancers such as gastric, breast, and bladder cancers, telomerase activity has been shown to be correlated with clinicopathological features of tumors such as clinical stage and pathological grade, which are both parameters of critical importance in determining the prognosis (11, 13, 15). In the case of neuroblastoma, telomerase activity levels have also been found to mirror disease outcome, whether regression or progression (16). These correlations might be explained by a selective growth advantage of cells expressing telomerase due to the improved stability of chromosomes with restored telomeres. The present study provided evidence that telomerase activity is associated with cellular proliferation. The previous study by Albanell et al. (23) obtained similar findings (that telomerase activity is down-regulated during tumor cell differentiation, and an inverse relationship exists between the degree of cellular differentiation and telomerase activity). These findings might also more directly explain the correlation between telomerase activity and the malignant potential of tumors.

The mechanisms by which telomerase activation is linked to cellular proliferation are unclear. Using an in vitro mouse skin carcinogenesis model, Bednarek et al. (24) demonstrated that a progressive

<table>
<thead>
<tr>
<th>PCNA activity</th>
<th>Telomerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative&lt;sup&gt;d&lt;/sup&gt; (n = 23)</td>
<td>9</td>
</tr>
<tr>
<td>Low&lt;sup&gt;e&lt;/sup&gt; (n = 5)</td>
<td>1</td>
</tr>
<tr>
<td>High&lt;sup&gt;f&lt;/sup&gt; (n = 14)</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Positive using 5 μg of extract, but negative using 0.5 μg of extract.
<sup>b</sup> Positive using 5 μg or 0.5 μg of extract, but negative using 0.05 μg of extract.
<sup>c</sup> Positive using 5 μg, 0.5 μg, or 0.05 μg of extract.
<sup>d</sup> Less than 5% positive cells.
<sup>e</sup> More than 5% but less than 30% positive cells.
<sup>f</sup> More than 30% positive cells.
increase in telomerase activity is associated with increased genomic instability. Hiyama et al. (16) have also reported that telomerase expression in neuroblastoma is often associated with genetic changes such as N-myc amplification and L-myc loss of heterozygosity. It therefore seems that telomerase activation is often associated with genetic alterations, which might lead to cellular proliferation. However, this is not the case with normal endometrium, because it contains no genetic alterations. Alternatively, because telomerase activity seems to be linked to cell differentiation, and the phenotypic changes concomitant with cell differentiation are linked to changes in the expression of important growth-regulation genes including transcription factors, oncogenes and tumor suppressor genes, and growth factors or their receptors (25-27), telomerase activity may involve these growth-regulating mechanisms. At present, we cannot exclude the possibility that cell proliferation secondarily activates telomerase expression. In this case, it is possible that factors regulating cell proliferation affect gene expression or the function of telomerase.

The present study also suggests the possibility that telomerase activity is regulated by sex steroid hormones. High telomerase activity was observed in the proliferative phase, whereas a marked reduction in activity was found in the secretory phase. The atrophic endometrium of postmenopausal women exhibited no or low telomerase activity, but atrophic endometrium treated with estrogen or tamoxifen exhibited moderate telomerase activity. These findings suggest that estrogen may activate telomerase expression, whereas progesterone may repress it. We do not know at present whether this hormone-dependent regulation of telomerase activity is confined to the endometrium.

The significance of the regulation of telomerase during the menstrual cycle is unclear. Normal endometrium in women of reproductive age repeats regeneration and withdrawal processes every 28 days. This rapid cycle of endometrial growth must be maintained by efficient growth-regulating machinery. Telomerase activity, which is correlated with the proliferative activity of endometrial cells, seems to involve this machinery and to contribute to the growth and withdrawal processes in normal endometrium during the menstrual cycle. Understanding the functional significance of telomerase expression in endometrial cells might provide important insight into the mechanisms of cell proliferation and cancer development.

Acknowledgments

We thank Dr. S. Okabe (Department of Obstetrics and Gynecology, Kanazawa National Hospital) for collecting samples. We also thank Dr. H. Hirano (Nippon Gene Co., Ltd.) for useful technical advice and discussions.

References

Telomerase Activity in Human Endometrium

Satoru Kyo, Masahiro Takakura, Takafumi Kohama, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/4/610

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.