Telomerase activity is found in the epithelial cells but not in the stromal cells in human endometrial cell culture

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Telomerase activity is associated with the proliferative activity of cells. In the endometrium, telomerase activity is higher in the proliferative phase than in the secretory phase of the menstrual cycle, suggesting that telomerase activity may occur primarily in the glandular epithelial cells. To test this, a dissociated cell culture of the endometrium was performed, and the telomerase activity in each cell fraction was analysed. Telomerase activity was found in all 10 endometrial tissues of the proliferative phase of the menstrual cycle. Both the fragments of epithelial glands and single cells, which were prepared by enzymatic dissociation, showed telomerase activity. In the 7 day cell culture, it was found in nine out of 10 epithelial cell enriched fractions, but in none of the stromal cell enriched fractions. Flow cytometric analysis showed that the epithelial cell enriched fraction was contaminated with a predominant number of stromal cells, while the stromal cell enriched fraction was comprised mostly of stromal cells with apparent proliferative activity. Our results suggest that telomerase activity of the endometrium occurs primarily in the epithelial cells in the endometrium and that the stromal cells do not express telomerase activity regardless of their potent proliferative activity.

Key words: cytokeratin/endometrium/epithelial cells/stromal cells/telomerase

Introduction

Telomerase is a ribonucleoprotein which protects the telomere from shortening. Telomerase activity has been determined in various tissues and cell lines during past years and >90% of cancer cells, as well as germ cell lines, and some somatic cells including the endometrium have been shown to express telomerase activity (Lundblad and Wright, 1996). In most normal somatic tissues, telomerase is repressed until the early stage of embryogenesis (Ulaner and Giudice, 1997) and its reactivation is considered to be a significant step in the carcinogenesis of cells (de Lange, 1994).

In immortal cells, telomerase activity is associated with the cell cycle (Holt et al., 1996). Highest telomerase activity is found during the S phase of the cell cycle (Zhu et al., 1996; Kruk et al., 1997), while quiescent cells in the G0 phase do not possess telomerase activity at a detectable level. In the endometrium, higher telomerase activity is found in the proliferative phase of the menstrual cycle (Kyo et al., 1997; Saito et al., 1997; Yokoyama et al., 1997). The endometrium is composed of multi-cellular components. The proliferative potentials of the individual endometrial cellular components are different during the menstrual cycle (Li et al., 1993). The epithelial cells of the endometrium show extremely high proliferative activity during the proliferative phase, while the stromal cells show persistent proliferative activity throughout the menstrual cycle with a slight increase in the late proliferative and late secretory phases (Jurgensen et al., 1996). These facts suggest that telomerase activity in the endometrium is associated with the proliferative activity of the epithelial cells.

Using a dissociated cell culture, we studied the telomerase activity in the epithelial cells and stromal cells of the endometrium in order to test this hypothesis.

Materials and methods

To collect proliferative endometria, patients whose menstrual date was earlier than the 15th day of the menstrual cycle were chosen. The endometria (10 samples) were obtained from the uterus of the patients who underwent surgery for leiomyoma of the uterus and/or pelvic endometriosis. The mean age of the patients was 42.0 years (range 36–48, SD 5.7). All patients had regular menstruation. None of the patients were administered oestrogenic and/or progestational agents before the operation. The endometrium was dated by histological findings according to Noyes et al. (1950).

Tissue dissociation and cell culture

The procedure for the cell dissociation culture was based on previous reports (Bongso et al., 1988; Matthews et al., 1992) with some modifications in the culture medium and culture dishes in order to augment the growth of the epithelial cells.

For the epithelial cell culture, culture dishes (95 mm in diameter, Falcon) were coated with poly-L-lysine in accordance with a previous report (Mahfoudi et al., 1991). Briefly, the culture dishes were incubated at room temperature with poly-L-lysine (5 mg/cm²) prepared in sterile distilled water. After 15 min, excess poly-L-lysine was removed, and the dishes were washed three times with sterile deionized water and air-dried.

For the epithelial cell culture or stromal cell culture, minimum essential medium (MEM) in which D-valine was substituted for L-valine (Cat# 12570: Gibo BRL, Rockville, MD, USA) or MEM essential medium (MEM) was used.
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(Gibco BRL) was used with 15% dialysed fetal bovine serum (FBS; Gibco BRL).

The endometrium was aseptically separated from the resected uterus and placed into Hank's balanced salt solution (HBSS; Gibco BRL) at 4°C. The tissues were minced into small pieces. Some pieces were fixed in 10% buffered formalin and submitted to histological diagnosis for endometrial dating.

For cell culture, the small pieces were washed in several changes of HBSS containing amphotericin B (5 µg/ml), streptomycin (100 µg/ml) and penicillin (100 IU/ml). The tissue pieces were further minced and then incubated for 2 h in 0.25% (w/v) collagenase (Type A; Sigma Chemical Company, St Louis, MO, USA) made up in HBSS with intermittent agitation at 37°C.

The cell suspension produced by collagenase digestion consisted of single stromal cells and fragments of epithelial glands. As a first step, these two populations were separated by filtration through a 45 µm nylon mesh. The fragments of the epithelial glands retained on the filter mesh were washed off with HBSS, collected by centrifugation, and suspended in the culture medium for the epithelial cell culture. They were seeded on the poly-l-lysine-coated culture dishes. This cell culture fraction was designated as the epithelial cell enriched fraction.

The cells that passed through the filter were collected by centrifugation and further filtered through a 10 µm nylon mesh. The cells that passed through this second filter were centrifuged and suspended in phosphate-buffered saline (PBS). They were overlayed on Percoll (Pharmacia, Milton Keynes, UK) and centrifuged for 15 min at 1000 g to remove the red blood cells. Cells were recovered, suspended in the culture medium for stromal cell culture and seeded in non-coated culture dishes. The cell fraction of this culture was designated as the stromal cell enriched fraction.

The entire medium was changed after 24 h. Cell growth was monitored every day, and the cells were recovered on the seventh day after seeding.

**Analysis of cell components in each cell fraction**

For immunohistochemical study of vimentin and/or cytokeratin expression, monoclonal mouse anti-vimentin antibody (Biomedia Corporation, Foster City, CA, USA) and rabbit polyclonal anti-cytokeratin antibody (Zymed Laboratory Inc, San Francisco, CA, USA) were used. Cells were fixed with methanol, incubated with these antibodies and subsequently Texas Red-labelled anti-mouse immunoglobulin G (IgG; Oncogene Science Inc, Uniondale, NY, USA) and fluorescein-labelled anti-rabbit IgG (Oncogene Science Inc).

For flow cytometric analysis of the ratio of cells with positive cytokeratin staining, the cells were incubated with 50-fold diluted rabbit polyclonal anti-cytokeratin antibody (Zymed Laboratory Inc) for 1 h in PBS, washed twice with PBS, incubated with fluorescein-labelled anti-rabbit IgG (200-fold dilution) for a further 30 min and then rinsed three times with PBS. The ratio of cells labelled with fluorescein was determined using an Epics Profile Analyzer.

For the cell cycle analysis, the cells were fixed in 70% ethanol at room temperature for 30 min. Cells were mixed with 10 µg/ml ethidium bromide and RNase A 50U/ml in PBS, incubated for 30 min, and washed twice with PBS. The emission of ethidium bromide was analysed by an Epics Profile Analyzer. Each fraction of the cell cycle phase was analyzed with Elite Software (Miami, FL, USA) based on the obtained histogram. Flow cytometric analyses were performed in triplicate.

**Telomerase detection assay**

Cultured cells (~1.0×10^6 cells) were washed once with PBS and scraped into a washing buffer (10 mM HEPES KOH, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, and 1 mM dithiothreitol). Endometrial tissues (~100 mg) were washed twice in PBS. The cells and tissues were then homogenized in 200 µl of a cell lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM MgCl₂, 1 mM ethylene glycol-bis (β-aminéthyl ether)N,N,N’,N’-tetraacetic acid (EGTA), 0.1 mM benzamidine, 5 mM β-mercaptoethanol, and 0.5% 3-(3-cholamidopropyl) dimethyl-ammonio)-1-propanesulphonate (CHAPS; Wako Chemical Industries Inc, Osaka, Japan), and 10% glycerol, and incubated on ice for 30 min. Cell homogenates were then centrifuged at 12 000 g for 20 min at 4°C. The supernatant was recovered, snap-frozen in liquid nitrogen and stored at –80°C.

The TRAP assay was performed using a TRAPEZE Telomerase detection kit (Oncor Inc, Gaithersburg, MD, USA). Briefly, 2 µl of tissue extract, and 48 µl of the TRAP reaction mix, consisting of 5’-end-labelled TS Primer (AACTGGTCGAGCAGAGTT) with γ-[32P]-ATP, 50 µM of dNTP mix, a TRAP primer mix (RP primer, K1 primer, and TSK1 template) and 2 IU Taq DNA polymerase in 20 mM Tris–HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 1 mM EGTA, 0.05% Tween 20, and 0.01% BSA, were mixed and incubated at 30°C for 30 min. Polymerase chain reaction (PCR) was then carried out as follows; 94°C, 30 s; 60°C, 30 s for 25 cycles.

PCR products were electrophoresed in 12% acrylamide gel and autoradiographed. As a positive and negative control, Ishikawa cells, cultured endometrial carcinoma cells, and the cell lysis buffer were used, respectively.

**Results**

Of the 10 samples examined in this study, all crude tissue samples of the endometrium expressed telomerase activity (Table I). In the cell culture, however, telomerase activity was found only in the epithelial cell enriched fraction and the positive rate was 90% (nine out of 10 samples examined). When compared with the telomerase activity of Ishikawa cells, the epithelial cell enriched fraction showed a much weaker telomerase activity (Figure 1). An immunofluorescent staining study revealed that stromal cells were cytokeratin-negative and vimentin-positive, whereas the epithelial cells were both cytokeratin- and vimentin-positive. Therefore, flow cytometric cell sorting was performed on the basis of the cytokeratin immunoreactivity. Upon flow cytometric analysis, in the epithelial cell enriched fraction, the percentage of cytokeratin-positive cells was 28.4–69.2%. In the one telomerase-negative case, of the epithelial cell enriched fraction, the number of cytokeratin-positive cells was 28.4%.

In order to rule out the possibility that undetectable telomerase activity in the stromal cell fraction was due to an accumulation in the G0 phase of the cell cycle, as a result of contact inhibition, the cell cycle of the stromal cell enriched fraction was analysed in cases 3, 6, and 8. In these cases, the number of cells in the S phase was found to be 11.3 (Figure 2), 14.6 and 13.8% of the whole population respectively.

Since telomerase activity was not found in some epithelial cell enriched fractions, we analysed the time course of telomerase activity of the epithelial cell enriched fraction in case no. 3. Immediately after collagenase digestion (Day 0), both cell fractions showed telomerase activity, but the epithelial cell enriched fraction apparently expressed higher telomerase activity.
Endometrial epithelial cells express telomerase activity

Table I. Telomerase activity and percent of cytokeratin-positive cells in cellular fractions of uterine endometrium

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Menstrual cycle day</th>
<th>Tissue telomerase activity</th>
<th>Epithelial fraction Telomerase activity</th>
<th>Percentage cytokeratin-positive (SD)</th>
<th>Stromal fraction Telomerase activity</th>
<th>Percentage cytokeratin-positive (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>69.2 (5.2)</td>
<td>−</td>
<td>6.7 (0.5)</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>65.3 (3.7)</td>
<td>−</td>
<td>5.8 (0.4)</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>58.1 (5.8)</td>
<td>−</td>
<td>7.6 (0.7)</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>54.9 (3.4)</td>
<td>−</td>
<td>6.2 (0.4)</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>13</td>
<td>+</td>
<td>+</td>
<td>47.7 (3.3)</td>
<td>−</td>
<td>8.4 (0.7)</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>11</td>
<td>+</td>
<td>+</td>
<td>45.4 (4.7)</td>
<td>−</td>
<td>5.3 (0.4)</td>
</tr>
<tr>
<td>7</td>
<td>44</td>
<td>9</td>
<td>+</td>
<td>+</td>
<td>39.9 (3.9)</td>
<td>−</td>
<td>6.2 (0.3)</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>36.5 (4.2)</td>
<td>−</td>
<td>5.3 (0.4)</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>29.7 (2.4)</td>
<td>−</td>
<td>5.9 (0.7)</td>
</tr>
<tr>
<td>10</td>
<td>46</td>
<td>9</td>
<td>+</td>
<td>−</td>
<td>28.4 (6.1)</td>
<td>−</td>
<td>7.1 (0.6)</td>
</tr>
</tbody>
</table>

Figure 1. Telomerase activity in epithelial cell enriched fractions (E) and stromal cells (S) enriched fraction. Telomerase activity is found only in epithelial cell enriched fractions. IS = internal standard; E = epithelial cell enriched fraction; S = stromal cell enriched fraction; P = positive control; N = negative control.

Once the stromal cell enriched fraction was put into the cell culture, telomerase activity was not found even in the 3 day culture. On the contrary, the epithelial cell enriched fraction expressed telomerase activity for at least 14 days, as shown in Figure 3.

Discussion

In normal somatic cells, the only cells which express telomerase activity are those which possess an extensive proliferative potential, such as hair follicle cells (Ramirez et al., 1997), keratinocytes (Yasumoto et al., 1996), endothelial cells (Hsiao et al., 1997), and cells of the deep mucosa in the stomach (Kuniyasu et al., 1997). This, taken together with the fluctuation of telomerase activity in immortal cells during the cell cycle, suggests that the expression of telomerase activity has a significant association with the proliferative activity of cells, regardless of their phenotype.

The endometrium is comprised of multicellular components. As well as epithelial cells and stromal cells, there are a considerable number of lymphocytes in the endometrium which exhibit proliferative activity throughout the menstrual cycle. The proliferative activity of these cells markedly increases in the secretory phase (Tabibzadeh, 1990). Lymphocytes are known to express telomerase activity (Pan et al., 1997). In addition to them, other telomerase-positive cells such as leukocytes (Buchkovich and Greider, 1996) and endothelial cells (Hsiao et al., 1997) exist in the endometrium.

Collagenase A treatment of the endometrium releases stromal cells as well as hematopoietic cells from the collagen matrix, producing a single cell population. We found weak but obvious telomerase activity in this single cell population.
We carefully and elaborately separated single cells from the glands of the endometrium after collagenase treatment, but the epithelial cell enriched fraction was contaminated with a number of stromal cells. To reduce the contamination in each cell fraction, especially in the epithelial cell enriched fraction, poly-L-lysine-coated culture dishes (Mahfoudi et al., 1991) and a medium containing d-valine (Gilbert and Migeon, 1975; Frauli and Ludwig, 1987) were available. We adapted both attempts, but we could obtain only a maximum of ~70% cytokeratin positivity in the epithelial cell culture. In contrast, in the stromal cell enriched fraction, >90% of the cells were cytokeratin-negative.

The epithelial enriched fraction showed positive telomerase activity, although this activity was much less than that of Ishikawa cells which were used as a positive control. The cells of this fraction are non-immortalized cells in the primary culture and thus undergo a limited number of cell divisions. In addition, this cell fraction was not comprised solely of epithelial cells. These points may explain the relatively weak telomerase activity in this cell fraction. On the contrary, telomerase activity was not found in the stromal cell enriched fraction, regardless of its potent proliferative activity. This result suggests that stromal cells of the endometrium do not principally express telomerase activity. The fact that only a limited number of somatic tissues possess telomerase activity, in spite of the widespread presence of stromal cell in the whole body, further supports our observation.

Although purified by filtration and Percoll gradients, this cell fraction would have been contaminated with several kinds of cells, including lymphocytes. Lymphocytes, however, cannot adhere to the non-treated surface of culture dishes. In addition, epithelial cells would have contaminated this cell fraction to some extent. These cells were mechanically released from epithelial glands, therefore some would not be intact enough to adhere to the culture dishes and to proliferate in culture.

Enzymatic dissociation and subsequent cell culture might influence the telomerase expression of the cells. The stromal cells, however, proliferate well in culture for a relatively long period, whereas epithelial cells can hardly maintain their proliferative activity in culture. Our observation of no negative telomerase activity in stromal cells is based on the cell culture. Even though these manipulations might affect telomerase status of the cells, our observation strongly suggests that stromal cells of the endometrium do not express detectable levels of the telomerase activity, or at least express it much less than epithelial cells.

References


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