Telomerase Activity in Hodgkin’s Disease

By Karl-Fredrik Norrback, Gunilla Enblad, Martin Erlanson, Christer Sundström, and Göran Roos

Telomere maintenance executed by the action of telomerase seems to be a prerequisite for immortalization. Telomerase is found in most cell lines and malignant tumors. A telomerase-independent mechanism for telomere maintenance in Hodgkin’s disease has been proposed in the absence of detectable telomerase activity. In this study, telomerase activity was detected in 31 of 77 Hodgkin’s disease samples and a strong correlation between eosinophilia and absence of detectable telomerase activity was found. Purified eosinophils and specifically eosinophil-derived neurotoxin and eosinophilic cationic protein, both ribonucleases, were found to degrade telomerase. Purified neutrophils also exhibited weak telomerase degradative activity. Reanalysis of previously telomerase-negative Hodgkin’s disease samples with stronger activity.9,10 In vitro activated T and B lymphocytes can upregulate telomerase activity, 9,11 and we have recently found telomerase activity in Hodgkin’s disease cell lines. Based on our data, Hodgkin’s lymphomas are potential targets for antitelomerase therapy.

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THE TELOMERIC ENDS consist of DNA repeats, in humans (TTAGGG)ₙ, and associated proteins with functional relevance for the integrity of chromosomes, chromosomal localization, and gene expression.1-3 Because of the properties of DNA-polymerase, lagging strand synthesis is incomplete, leading to a subsequent shortening of the telomeres with each DNA replication round, a phenomenon called the end replication problem.4 This problem precludes infinite growth unless the telomeric ends are reconstituted. At critical telomere shortening, normal somatic cells undergo senescence.5 In vitro, a strong association between telomere length and induction of senescence has been demonstrated, and the telomere has been proposed to act as a molecular clock for cell proliferation.5,6 In most permanently growing cell lines, as well as in a majority of tumors, telomerase activity seems to compensate for the telomere loss upon cell division by adding new telomeric repeats to the chromosome ends.7,8 The activity of telomerase seems to be a prerequisite for an infinite life span, although other mechanisms for telomere maintenance probably exist.7

Within the hematopoietic system, peripheral blood cells have been demonstrated to express weak telomerase activity, whereas early progenitor stem cells in the bone marrow seem to have stronger activity.9,10 In vitro activated T and B lymphocytes can upregulate telomerase activity,9,11 and we have recently found strong activity in normal germinal center B cells indicating that, in vivo, a significant upregulation of telomerase can occur.12

There are principally two ways that a tumor cell might acquire telomerase activity.13,14 One is reactivation of telomerase through a process induced by critical telomere shortening. The second pathway is through retention of the ability to express telomerase activity from a normal cell competent to upregulate telomerase, like bone marrow stem cells and lymphocytes. We believe that the latter is the main pathway hematological malignancies use to acquire telomerase activity.9 Retention of telomerase activity was supported by data in non-Hodgkin’s lymphomas that at diagnosis had significantly increased telomerase activity levels compared with reactive lymph nodes.12

Hodgkin’s disease (HD) is subclassified into different subgroups based on histopathological appearance and the number of Hodgkin’s and Reed-Sternberg (H-RS) cells. A typical feature of HD, although missing in some cases, is infiltration of eosinophils. The tumor component of HD, ie, the H-RS cells, constitutes only 1% to 2% of all cells.15 Because of the proliferative demands on the clonal H-RS cells, which are actively cycling, they are expected to exhibit mechanisms for maintenance of telomere integrity.6,16,17 In a previous study, a telomerase-independent maintenance mechanism of the telomeres in HD was proposed in the absence of detectable telomerase activity.18

In the current study, we have analyzed telomerase activity using a quantitative technique in 77 HD lymph nodes and in HD-derived cell lines. In contrast to the previous report, we could demonstrate telomerase positivity in 80% to 90% of the HD samples, and the level of telomerase expression was increased compared with reactive lymph nodes. All HD-derived cell lines expressed high levels of telomerase. Eosinophilia, specifically the ribonuclease (RNase) content of the eosinophils, was the probable reason for artifactual telomerase negativity previously reported in HD.19

MATERIALS AND METHODS

Patient samples. Seventy-seven lymph nodes with HD were obtained as frozen tissue stored at −80°C. All samples were diagnostic lymph nodes and the patients were untreated during the 3 months before sampling. The histopathological diagnoses were reexamined in a blinded manner by one of the authors (C.S.) using the Rye classification. Among the 77 Hodgkin’s lymphomas, 45 cases were classified as nodular sclerosis (NS), 24 cases as mixed cellularity (MC), 4 cases as lymphocytic predominance (LP), and 1 case as lymphocytic depletion (LD) subtype. Three cases were unclassifiable.

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Four high-grade malignant and 5 low-grade malignant non-Hodgkin’s lymphomas classified according to the Kiel classification as well as 15 reactive lymph nodes were also added to the study as reference cases. All reference samples were diagnostic and derived from lymph nodes.

**Cell lines.** Primary foreskin fibroblasts and the following established cell lines were used in the study: T47D1 (breast carcinoma), Hela (epithelial carcinoma of the cervix; obtained from the American Type Culture Collection, Rockville, MD), and HDLM-2 (HD-derived), KM-H2 (HD-derived), and L-428 (HD-derived) obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The 1301 (T-cell lymphoblastic) cell line was a kind gift from Prof Erik Lundgren (Department of Cell and Molecular Biology, Umeå University, Umeå, Sweden).

**S-phase determination.** Fresh cells in suspension were used for DNA staining according to Vindelov et al., and the flow cytometric analysis was performed using a FACSscan instrument (Becton Dickinson Immunocytometry Systems, San José, CA). S-phase fractions were calculated using the Cellfit software using the RFTT evaluation model (Becton Dickinson).

**Purified cell populations.** Pure populations of eosinophilic and neutrophilic granulocytes were prepared as described and were a kind gift from Dr Per Venge (Department of Clinical Chemistry, Uppsala University, Uppsala, Sweden). Briefly, blood from normal donors was centrifuged over an isotonic Percoll solution and the mononuclear cell fraction was removed. The pellet containing neutrophils, eosinophils, and erythrocytes was then resuspended and the red blood cells were lysed. The remaining cells were incubated with anti-CD16 magnetic particles and separated using the Macs cell separation system (Miltenyi Biotec Inc, Auburn, CA). After the nonlabeled CD16+ eosinophils had been eluted, the separation column was removed from the magnetic field and the CD16-labeled neutrophilic granulocytes were eluted.

**Purified RNases.** Purified eosinophil-derived neutroxin (EDN) and eosinophil cationic protein (ECP) were kind gifts from Dr Per Venge. EDN and ECP, which are RNases found at high concentrations in eosinophilic granulocytes, were purified to homogeneity from buffy coats obtained from healthy individuals, as previously described.

**Counting of cells.** The number of eosinophils and H-RS cells was counted on hematoxylin–eosin–stained paraffin sections by one of the authors (G.E.), as described. The cells were counted in 10 randomly selected high-power vision fields. To facilitate counting, an eye-piece equipped with a lattice framework were counted. In cases with nodular sclerosis, cellular regions were counted. The presence or absence of germinal centers and necroses was also noted for each specimen.

**Telomerase assay.** Cellular extracts were prepared from tissue pieces or frozen sections (3 × 40 µm) by adding an appropriate volume of lysis buffer. The homogenate was stored on ice for 30 minutes and centrifuged at 13,000g for 20 minutes at 4°C. The supernatant was collected and snap-frozen in liquid nitrogen before storage at −80°C. Protein measurements were performed using the BCA protein assay kit (Pierce Chemical Co, Rockford, IL), and the extracts were diluted to a final concentration of 0.14 µg/µL. A few reactive nodes and non-Hodgkin’s lymphomas were prepared as fresh samples from which cell suspensions were made and extracts prepared at a cell number/mlcorresponding to a protein concentration of 0.14 µg/µL, as described.

The TRAP (telomeric repeat amplification protocol) assay was performed as described. Quantification of telomerase activity levels was made possible with inclusion of the internal telomerase assay standard (ITAS) used at 15 attograms/assay. The polymerase chain reaction (PCR) products were resolved on a 10% nondenaturing polyacrylamide gel with the ITAS separating above the telomerase products. After fixation the gels were mounted on Phosphorimager screens and were thereafter analyzed in a Molecular Imager system GS-525 using the Phosphor Analyst version 1.4.1 (Bio-Rad Laboratories, Hercules, CA). The telomerase activity level of a sample is the mean of two or more analyses at 0.28 µg protein/assay described to be within the linear range of the TRAP assay (Norrback et al, unpublished data). The telomerase activity was defined as the ratio between the telomerase products formed and the ITAS product corrected for background. Lysis buffer served as negative control and background. No marked differences were observed if subtraction was performed for background levels within each lane above the PCR products. A sample was considered telomerase positive when a clear amplification of ITAS was observed and the telomerase activity exhibited high processivity. For an extract to be considered telomerase negative, no telomerase products were visible, and the ITAS had to be amplified greater than 90% of the ITAS in the lysis buffer to exclude the possibility of Taq inhibition or RNase activity in the sample. RNase incubation of selected samples verified that genuine RNA-dependent telomerase products were being formed.

**Additional chemicals.** Additional chemicals used during the course of the study were rRNasin (placental RNase inhibitor; Promega Corp, Madison, WI), dithiothreitol (DTT; Sigma Chemical Co, St Louis, MO), high purity grade RNase DNSTase-free (Boehringer Mannheim Gmbh, Mannheim, Germany), and RNase A (US Biochemical, Cleveland, OH).

**Statistical methods.** Correlation between different variables was tested according to Spearman’s test. Differences between groups were tested with Mann-Whitney’s rank sum test or Kruskal-Wallis’ test. The χ² test was used when comparing the proportions of different groups.

**RESULTS**

Thirty-one of 77 HD samples (40%) were found to be telomerase positive. Thus, a majority of the samples were devoid of detectable telomerase activity. Storage time of the HD samples or preparation technique had no impact on the level of telomerase activity. Also, the telomerase activity levels could not be shown to be affected by the presence of necrosis or germinal centers. However, a striking correlation between the presence of eosinophilic granulocytes and the absence of telomerase activity was found (P < .00001, Table 1). When extracts from telomerase-negative HD cases with moderate eosinophilia were mixed with extracts from a telomerase-positive cell line (T47D1, 0.28 µg/assay) at room temperature (RT) before the TRAP assay, the telomerase activity of the cell line was abolished.

To elucidate whether the telomerase-inhibitory activity of the HD samples was due to the eosinophils per se or some other factor involved in the eosinophilia, eosinophilic granulocytes were purified (purity, 99%). When Hela cell extracts were preincubated with an extract corresponding to 50 eosinophils the TRAP assay, the telomerase activity of the cell line was abolished.

**Table 1. Relationship Between Eosinophilia and Telomerase Activity in HD Samples**

<table>
<thead>
<tr>
<th>Eosinophils*</th>
<th>Fraction of Telomerase-Positive Cases</th>
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<tbody>
<tr>
<td>0</td>
<td>24/27 (88.9%)</td>
</tr>
<tr>
<td>1-10</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>11-200</td>
<td>1/29 (3.4%)</td>
</tr>
<tr>
<td>&gt; 200</td>
<td>1/12 (8.3%)</td>
</tr>
</tbody>
</table>

The correlation between the presence of eosinophils and the absence of telomerase activity was highly significant (P < .00001). *Number of eosinophils counted in 10 randomly selected high power vision fields. The eosinophils were not counted in 1 HD sample. †The HD sample that exhibited detectable telomerase activity in the category > 200 eosinophils was the 1 lymphocyte depletion (LD) case of the whole study.
extract corresponding to 500 purified neutrophils (purity, 99.8%) was also shown to abolish the telomerase activity of the Hela cell line but was much less potent than the eosinophil extracts (Fig 1). Considering that EDN and ECP possess RNase activity and are present in high amounts in eosinophils and to a much lesser degree in neutrophils,19,29 we added placental RNase inhibitor to the experiments described above.30 The RNase inhibitor was used at a concentration of 1 U/µL or 10 U/µL together with 1 mmol/L DTT. When the RNase inhibitor was present in mixed extracts containing a telomerase-positive cell line and purified eosinophils or neutrophils, the telomerase activity of the cell line was always rescued (Fig 1).

To prove that EDN and ECP were the enzymes responsible for the degradation of telomerase, we repeated similar experiments with purified EDN and ECP. If 0.2 ng EDN was preincubated together with the Hela cell line (0.28 µg/assay) for 20 minutes at RT before the TRAP assay, the telomerase activity of the cell line was almost completely lost. Ten times less EDN was needed to abrogate the telomerase activity of the cell line if the preincubation time was extended (Fig 2). EDN was more potent than ECP at degrading telomerase, reflecting the difference in potency of the RNases.29 Commercially bought RNases showed roughly similar kinetics of telomerase degradation. The presence of placental RNase inhibitor in mixtures containing a telomerase-positive cell line and pure RNases always rescued the telomerase activity (Fig 2). The experiments investigating the telomerase degradative activity of granulocyte extracts and purified RNases were verified using the cell lines Hela, T47D1, and 1301.

Finally, we prepared new extracts with the placental RNase inhibitor present from 6 previously telomerase-negative HD samples exhibiting moderate eosinophilia, and 5 samples expressed clearly detectable telomerase activity (Fig 3). When purified eosinophilic and neutrophilic granulocytes were reanalyzed using the RNase inhibitor, they remained telomerase-negative.

Unexpectedly, when RNase-containing cell extracts were analyzed with the TRAP assay, the amplification of ITAS was lost or diminished (Figs 1 and 4). The presence of the placental RNase inhibitor in these extracts was found to always rescue the ITAS amplification. The pure RNases by themselves did not repress the amplification of ITAS. In contrast, when the RNases were preincubated with cell extracts of telomerase-positive cell lines or telomerase-negative fibroblast cultures at RT before the TRAP assay, the amplification of ITAS was lost (Fig 2). The presence of the RNase inhibitor in the mixed extracts similarly rescued the ITAS amplification. This effect of RNase-containing cell extracts or pure RNases on the ITAS amplification was also seen at +37°C. We also performed assays to evaluate the presence of Taq inhibitors or deoxyribonuclease (DNase) activity without demonstrating positivity in the extracts or in the purified RNases.

Only the group of HD samples without eosinophilia was used for quantitative estimates of telomerase activity, and 24 of 27 cases (89%) clearly exhibited telomerase activity. All telomerase-positive HD cases had an undisturbed amplification of ITAS, and the 3 telomerase-negative cases fulfilled the conditions for true telomerase negativity. The level of telomerase activity in the Hodgkin’s lymphomas was significantly higher than in 15 reactive lymph nodes (P = .05, Table 2) and significantly lower than in 9 non-Hodgkin’s lymphomas (P = .001, Table 2). The H-RS cell counts did not correlate with the level of telomerase, and only small differences with respect to telomerase activity levels were observed between the HD subgroups (Table 2).
We also analyzed telomerase activity levels in the HD-derived cell lines, HDLM-2, KM-H2, and L-428 (Table 2 and Fig 4). All cell lines expressed significant levels of telomerase activity, but the more slowly proliferating HDLM-2 (S phase, 13.5%) expressed decreased levels compared with KM-H2 (S phase, 45.4%) and L-428 (S phase, 34.8%).

**DISCUSSION**

In the present study, telomerase activity was detected in 31 of 77 HD lymph nodes (40%), but a strong correlation between the presence of eosinophilia and the absence of telomerase activity was found. Using purified eosinophils, we could demonstrate that they harbored the telomerase degradative activity observed in the HD samples with eosinophilia. Extracts from purified neutrophils corresponding to high cell numbers were also shown to contain weak degradative activity. The telomerase degradative activity contained within both cell types could be inhibited by the addition of placental RNase inhibitor. Purified EDN and ECP, being our candidate enzymes, were shown to degrade telomerase activity with a potency matching that of the eosinophil and neutrophil extracts based on the content of these RNases in the cells. The activity of both EDN and ECP could similarly be inhibited by the addition of the RNase inhibitor. Reanalysis of previously telomerase-negative HD samples with eosinophilia using RNase protection resulted in detection of telomerase activity. Eosinophils thus have a considerable impact upon the detectability of telomerase in HD and, specifically, the activity of EDN and ECP was responsible for the degradation of telomerase. However, we cannot exclude the possibility that the eosinophils contained additional molecules that contributed to the telomerase degradative activity observed, because immunodepletion of the extract with antibodies against EDN and ECP was not performed.

Because of the potency of eosinophils to quench the telomerase signal, previous studies on materials in which the number of eosinophils could be increased might have resulted in false-negative results.
negatives. Similarly, the RNase content in myeloid leukemias and large infiltrations of neutrophils could have resulted in the detection of false telomerase activity values. We have also found that serum from normal blood donors, when mixed with a telomerase-positive cell line, resulted in diminished telomerase activity that could be rescued in the presence of the placental RNase inhibitor (Norrback et al, unpublished data). EDN, also known as nonsecretory urinary RNase and liver RNase, which is present in blood and urine together with other RNases, could thus be a source of false results when analyzing telomerase activity in these fluids. It needs to be clarified to which cells/tissues addition of exogenous RNase inhibitors is required to obtain standardized results when analyzing telomerase activity. The addition of RNase inhibitors to all assays detecting telomerase activity is, at present, highly recommended.

Polymorphonuclear cells, including neutrophils, are considered to be telomerase-negative based on a study not using RNase inhibitors. We have also found that serum from normal blood donors, when mixed with a telomerase-positive cell line, resulted in diminished telomerase activity that could be rescued in the presence of the placental RNase inhibitor (Norrback et al, unpublished data). EDN, also known as nonsecretory urinary RNase and liver RNase, which is present in blood and urine together with other RNases, could thus be a source of false results when analyzing telomerase activity in these fluids. It needs to be clarified to which cells/tissues addition of exogenous RNase inhibitors is required to obtain standardized results when analyzing telomerase activity. The addition of RNase inhibitors to all assays detecting telomerase activity is, at present, highly recommended.

To analyze HD samples with no eosinophils for quantitative estimates of telomerase activity due to the noncovalent binding of the placental RNase inhibitor to the RNases. Telomerase activity was detected in 89% (24/27) of the Hodgkin’s lymphomas without eosinophilia not using RNase protection. The Hodgkin’s lymphomas exhibited significantly higher levels of telomerase activity than did reactive lymph nodes and significantly lower levels than non-Hodgkin’s lymphomas. We thus show that HD forms an intermediate entity with respect to the level of telomerase expression. That the telomerase activity levels of the HD samples were correct was supported by the high fraction of positivity, the undisturbed amplification of the ITAS, and the fact that all negative samples fulfilled the conditions for true telomerase negativity. When previously telomerase-negative HD samples with eosinophilia were reanalyzed using RNase protection telomerase positivity (5 of 6) was observed, indicating that most Hodgkin’s lymphomas with eosinophilia were telomerase positive as well.

The H-RS cell counts did not correlate to telomerase activity

Table 2. Levels of Telomerase Activity in HD-Affected Lymph Nodes, Non-Hodgkin’s Lymphomas, Reactive Lymph Nodes, and in Permanent Cell Lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Telomerase Activity*</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>HD, all cases</td>
<td>0.58</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
<td>0.65</td>
</tr>
<tr>
<td>Mixed cellularity</td>
<td>0.47</td>
</tr>
<tr>
<td>Lymphocytic predominance</td>
<td>0.72</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma, all cases</td>
<td>1.81</td>
</tr>
<tr>
<td>High-grade malignant</td>
<td>2.80</td>
</tr>
<tr>
<td>Low-grade malignant</td>
<td>1.04</td>
</tr>
<tr>
<td>Reactive lymph node</td>
<td>0.30</td>
</tr>
<tr>
<td>Cell lines</td>
<td></td>
</tr>
<tr>
<td>HDLM-2 (HD-derived)</td>
<td>3.98</td>
</tr>
<tr>
<td>KM-H2 (HD-derived)</td>
<td>7.87</td>
</tr>
<tr>
<td>L-428 (HD-derived)</td>
<td>8.17</td>
</tr>
<tr>
<td>1301 (T cell lymphoblastic)</td>
<td>17.25</td>
</tr>
<tr>
<td>T47D1 (breast carcinoma)</td>
<td>1.37</td>
</tr>
<tr>
<td>Hela (cervical carcinoma)</td>
<td>2.95</td>
</tr>
</tbody>
</table>

*The level of telomerase activity was analyzed in extracts at 0.28 µg/assay without RNase protection using the TRAP assay and ITAS (see the Materials and Methods). The telomerase activity levels in HD were significantly increased compared with reactive lymph nodes (P < .05) and significantly decreased compared with non-Hodgkin’s lymphomas (P < .001).

†One case could not be subclassified.
levels in the HD cases without eosinophilia. The tumor component of HD constitutes only 1% to 2% of all cells, and reactive lymph nodes also express telomerase activity to a certain degree.2,15 Thus, a correlation between telomerase activity levels and tumor cell density might be difficult to find. The heterogeneity of the tumor component in HD could also undermine a correlation between H-RS cell counts and telomerase levels when all HD samples are studied as a group.31,39

The H-RS cells, which are clonal and cycling, would be expected to express telomerase activity to maintain the telomere integrity.16,17 The increased telomerase activity found in HD compared with reactive lymph nodes suggests that the H-RS cells express telomerase activity in vivo. The support is emphasized knowing that germinal centers, which are the sole source of high levels of telomerase expression in lymph nodes,12,40 were almost completely absent in the HD samples. The TRAP assay being PCR-based easily detects telomerase activity from 0.1% to 1% of telomerase-positive cells, making it reasonable to assume that the H-RS cells in vivo could contribute to the telomerase activity levels detected in the HD samples. The HD-derived cell lines chosen in this study, generally accepted to be derived from H-RS cells based on extensive characterization,31,39 all expressed significant levels of telomerase activity. The telomerase positive cell lines, which were derived both from MC and NS subtypes, possibly indicates a general ability of the H-RS cells to express the enzyme. It also suggests that H-RS cells maintain the telomere integrity by the action of telomerase and not by an alternative telomerase-independent mechanism recently described to exist in a minority of tumors and tumor-derived cell lines.7 There seemed to be a correlation between cell proliferation and the expression level of telomerase in the cell lines that would be expected because H-RS cells in many cases are derived from lymphocytes. However, further studies are required to prove the cell cycle connection to the expression of telomerase in H-RS cells.

An attractive future scenario within the field of hematology is a first-line treatment with cytotoxic drug(s), followed by antitelomerase treatment leading to telomere shortening of the tumor cells with subsequent cell death. A resistance factor to the actions of antitelomerase drug(s) would be the existence of an alternative telomerase-independent mechanism to maintain the telomeres. Such a mechanism was recently proposed to exist in HD in the absence of detectable telomerase activity.15 There is currently no support for the existence of a telomerase-independent mechanism for the telomere maintenance in HD. To date, the findings that HD lymph nodes and HD-derived cell lines express telomerase activity and the support for telomerase positivity of the tumor component in vivo renders HD a potential target for future antitelomerase therapy.

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