Telomerase Activation in Normal B Lymphocytes and Non-Hodgkin’s Lymphomas

By Karl-Fredrik Norrback, Katarina Dahlenborg, Roland Carlsson, and Göran Roos

Activation of telomerase seems to be a prerequisite for immortalization and is found in permanent cell lines and most malignant tumors. Normal somatic cells are generally telomerase negative, except for bone marrow stem cells. Weak activity is also present in peripheral blood cells. In the present study strong telomerase activity was demonstrated in vivo in normal mature cells of the immune system, as well as in malignant lymphomas. Benign lymph nodes had lower telomerase activity than benign tonsils, which exhibited intermediate high activity comparable with findings in malignant lymphomas. In benign tonsils the activity seemed to be restricted to germinal center B cells. In benign lymphoid tissues telomerase activity correlated with B-cell numbers and cell proliferation, but this was not observed in the lymphoma group. High-grade lymphomas exhibited higher levels of telomerase compared with low-grade cases. The data showed that in vivo activation of telomerase is a characteristic feature of germinal center B cells. Different signals for activation of telomerase are likely to exist, one of them being immune stimulation. The data suggest that telomerase activity in malignant lymphomas can be explained by an "induction and retention" model, ie, transformation occurs in a normal, mature B cell with reactivated telomerase, which is retained in the neoplastic clone.

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The Telomeres consist of highly conserved tandemly repeated G-rich sequences, in humans TTAGGG, with a total of 5 to 15 kb per chromosome end. The telomeric repeats with associated proteins are implicated in chromosome stabilization, spatial localization of chromosomes, and gene expression. In normal somatic cells increasing age and cell division number lead to a decreasing length of telomeres due to incomplete replication of the telomeric ends. In such cells senescence is induced in vitro at a critical minimum telomeric size, and the telomere has been proposed to act as a molecular clock for cell proliferation. Telomerase, a ribonucleoprotein first described in Tetrahymena, can compensate for the telomere loss by adding new telomeric repeats to the chromosome ends. When cells are immortalized in vitro, activation of telomerase occurs and this activity seems to be a prerequisite for an infinite life-span. However, a few exceptions from this rule have been found showing permanent cell lines without telomerase activity, indicating the possibility of an alternative mechanism for telomere length maintenance.

A large number of human tumors have been shown to contain active telomerase, whereas normal tissues as a rule have been negative. In peripheral blood (PB) cells weak activity has been detected, whereas specific bone marrow (BM) stem cell populations seem to have stronger telomerase activity. Mitogen stimulation of lymphocytes in vitro can induce activation of telomerase. Based on these data, and with the exceptions mentioned, telomerase activity seems to be a malignancy marker, which might provide a potential target for therapeutic intervention.

The mechanism(s) for activation of telomerase remains to be identified, but interaction with genes of importance for senescence and immortalization seems probable. Both the p53 gene and the retinoblastoma susceptibility (RB-1) gene have been indicated to be involved in senescence. Inactivation of p53 and RB-1 genes is associated with a prolonged life-span, but usually not immortalization. The process of immortalization is most likely due to loss of normal gene function, because most hybrids between normal and immortalized cells have a limited life-span. Hybrid experiments have identified at least four complementation groups for immortalization. In a recent report no correlation between telomerase activity and immortalization complementation group or between telomerase activity and abnormalities in RB-1, p53, and p16INK4a could be shown.

In the present study we asked if telomerase activity is a malignancy-associated property also for lymphoid cells and if activation occurs in normal cells in vivo as in vitro. A series of benign lymphoid tissues, malignant lymphomas, and cell lines were analyzed with respect to telomerase activity using the TRAP (telomeric repeat amplification protocol) assay. To our surprise strong telomerase activity was found in normal germinal center B cells (GC-B) at levels comparable with the activity demonstrated in malignant lymphomas and permanent cell lines.

Materials and Methods

Samples. Fifteen benign lymph nodes and 26 malignant non-Hodgkin’s lymphomas (NHLs) were obtained as frozen tissue stored at −80°C for a maximum of 2 years or as fresh samples. All the lymphoma samples were diagnostic, derived from lymph nodes and the patients were untreated. Twelve cases were high-grade malignant (HGM) and 14 cases were low-grade malignant (LGM) lymphomas according to the Kiel classification. Most cases were immunophenotyped and a majority of the samples were analyzed with respect to S-phase fractions.

Twenty-five benign tonsils were obtained from 15 patients undergoing tonsillectomy. PB was obtained from healthy blood donors.

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and normal BM samples were obtained from patients without any evidence of BM disease.

The following hematopoietic established cell lines were also tested. Avon (B-cell, lymphoblastoid), RPMI 8226 (myeloma), Namalwa (Burkitt’s lymphoma), Raji (Burkitt’s lymphoma), 1301 (T-cell lymphoblastic), Jurkat (T-cell lymphoblastic), K562-4 (erythroid), and U-937 (monoblastic).

**S-phase determination.** DNA staining was performed on fresh cells in suspension according to Vindelov et al. and the flow cytometric analysis using a FACScan instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA). S-phase fractions were calculated by the Cellfit software using the RFlT evaluation model (Becton Dickinson). Only diploid cases were evaluated regarding S-phase fractions.

**Immunophenotyping.** Samples were immunophenotyped using a panel of monoclonal antibodies (MoAbs) and flow cytometry. The B-cell fraction was determined as the percentage of CD19+ cells (CD19 antibody from Becton Dickinson) and as a criterion for monoclonality Ig light-chain restriction was used.

**Cell separations.** Separation into B-cell–enriched (CD19+) and B-cell–depleted (CD19−) cell fractions was performed on three benign tonsils from different donors using Dynabead M-450 Pan B (CD19) antibody and immunomagnetic sorting (Dynal A.S., Oslo, Norway). The purity of the fractions were evaluated using CD20 and CD3 antibodies (Becton Dickinson) and a FACScan flow cytometer (Becton Dickinson).

B-cell subpopulations were also prepared as previously described with some minor modifications to further analyze the B-cell compartment. Briefly, two benign tonsils from different donors were minced and prepared by density gradient Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) centrifugation. T cells were removed by two cycles of rosetting with neuraminidase treated sheep red blood cells as described. The T-cell–depleted fraction was separated into high-density (B0) resting cells and low-density cells on discontinuous Percoll gradient (60%, 65%) centrifugation. Cells banding above 60% and throughout this layer were submitted to additional negative selection with sheep-antimouse IgG (SAM) magnetic beads (Dynal A.S.) coated with antibodies to CD39 (AC2; kindly provided by M. Rowe, Cancer Studies, University of Birmingham, Birmingham, UK) and IgD to obtain GC-B cells. To further separate centroblast B cells (CB-B) from GC-B, the cell population was submitted to an additional depletion with SAM beads coated with anti-CD44 as described. Mouse anti CD44 (BU 52) was a generous gift from Prof I.C.M. MacLennan (Medical School, University of Birmingham, Birmingham, UK). High-density, resting B cells were collected as cells distributed throughout the 65% layer and pelleting below. All steps were done at 4°C except gradient centrifugations that were performed at room temperature. The cell fractions were evaluated by staining with antibodies directed to CD38, IgD, and CD19 (all from Becton Dickinson). Thus, resting B (B0) cells were defined as high density, CD19+, IgD+, CD38lo cells, GC-B as low density, CD39−, IgD+, CD19+, CD38lo cells and CB-B as low density, CD39−, IgD+, CD19−, CD44+, CD38lo cells.

**Telomerase assay.** All freshly collected samples were separated to receive a mononuclear cell suspension using anappropriate volume of lysis buffer. Protein measurements were performed using the BCA protein assay kit (Pierce Chemical Co, Rockford, IL). The frozen tissue extracts were diluted to a final protein concentration of 0.14 μg/μL.

The TRAP assay was performed as described. In addition, semi-quantitative analysis was performed, as exploited by others, in that a relative telomerase activity in percent of a reference cell line (1301) was determined for all samples. The TRAP assay is not completely linear; therefore, all samples were measured against the reference cell line at the equivalent cell number or protein content. Two microliters of the extracts from the freshly collected samples, equivalent to 1,000 cells, were run in parallel with 1,000 cells of the 1301 line. Extracts obtained from frozen tissue were run at a protein level of 0.28 μg per assay, roughly comparable to 1,500 cells based on control experiments in which specimens counted for cell numbers also were measured for protein, and were related to 1301 cell extracts run at the same amount of protein per assay. All samples were run in duplicates except for normal BM and PB. Taq polymerase activity was quantitated in a manner that allowed for the 1301 line.
polymerase was not limiting during the experimental conditions used as judged from control analyses using a recently described internal standard for the telomerase assay (Fig 1).24 To better control the time of the incubation step, where the elongation of the TS primer occurs, the time of adding the extracts to the reaction mixture was standardized and the 1301 extracts were added first and last in all pipetting series. RNase treatment was only performed on selected cases because it is our experience that it is easy to distinguish a truly telomerase-positive extract from artifacts.24

The polymerase chain reaction (PCR) products were resolved on a 10% nondenaturating polyacrylamide gel. After fixation the gels were exposed to Phosphorimager screens without drying for 75 and 225 minutes to check for linearity upon variable exposure times, and were thereafter analyzed in a Bio-Rad Molecular Imager using Phosphoranalyst version 1.1.1 (Bio-Rad Laboratories, Hercules, CA). A cell extract was considered as negative if no ladder was detectable after a 10-hour exposure. The telomerase-positive extracts were given a relative value of activity in percent of the reference cell line, based on the mean value of two separate runs with two different exposures. The ladder was framed and subtracted for background density levels in an equally sized rectangle. The negative control was used as background. No marked differences were obtained if subtraction was done for background levels within each lane above the ladders.

Statistical methods. The correlation coefficient r was calculated according to Pearson. To determine differences between two groups the Wilcoxon rank sum test was used.

RESULTS

It is desirable to run the TRAP assay at a low protein concentration because for extracts with high telomerase activity the Taq polymerase can limit the amount of PCR products formed. This is observed as a saturation of the incorporation of radioactive nucleotides.22,33 It was shown that Taq polymerase was not limiting at the two levels used of our reference cell line (Fig 1). It follows that the activity of telomerase in all samples that were related to the reference cell line could not have been limited by Taq polymerase because the activity of the cell line used always exceeded that of the samples. Inhibition of Taq polymerase has occasionally been observed in our laboratory, giving rise to
**Table 2. Telomerase Activity in Immunoselected Subpopulations of Lymphocytes From Benign Tonsils**

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Tonsil 1 (B)</th>
<th>Tonsil 2 (B)</th>
<th>Tonsil 3 (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cells</td>
<td>65.7</td>
<td>67.5</td>
<td>59.1</td>
</tr>
<tr>
<td>CD19-enriched</td>
<td>96.3</td>
<td>97.5</td>
<td>97.5</td>
</tr>
<tr>
<td>CD19-depleted</td>
<td>13.5</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Relative telomerase activity in percent of 1301 cell activity.
Table 3. Telomerase Activity in Immunoselected Subpopulations of B Lymphocytes From Benign Tonsils

<table>
<thead>
<tr>
<th>Cell Fraction*</th>
<th>Tonsil 4</th>
<th></th>
<th></th>
<th>Tonsil 5</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purity</td>
<td>Telomerase Activity (%)</td>
<td>Purity</td>
<td>Telomerase Activity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bn (resting B cells)</td>
<td>63</td>
<td>4.8</td>
<td>65</td>
<td>6.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-B (germinal center B cells)</td>
<td>86</td>
<td>40.2</td>
<td>93</td>
<td>79.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB-B (centroblasts)</td>
<td>96</td>
<td>30.5</td>
<td>95</td>
<td>49.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For information regarding the cell fractions, see Materials and Methods.
† Determined by flow cytometric analysis; see Materials and Methods.
‡ Relative telomerase activity in percent of 1301 cell activity.

activity ranging from 0.5% to 7.6% of 1301 cell activity (Fig 3, Table 1). Eight permanently growing hematopoietic cell lines were tested, all showing intermediate to high activity, except for one Epstein-Barr virus (EBV) transformed, lymphoblastoid cell line (Avon), which had been in culture for 4 months and demonstrated weak activity (1.5% of 1301 cell activity) (Fig 3, Table 1).

DISCUSSION

PB B cells exhibit no or very low levels of telomerase22,23 (and our unpublished data, 1995), indicating that mature B cells have lost the telomerase activity once present in the committed progenitor cells.23 In the present study telomerase reactivation was demonstrated in benign lymphoid tissues and in NHLs. The significant telomerase activity in benign lymphoid tissues seemed to be derived from the germinal centers as telomerase was almost solely expressed in GC-B cells from benign tonsils. Germinal centers are formed upon immune stimulation and this physiologic signal can obviously reactivate high levels of telomerase. The strongly telomerase-positive GC-B cells containing rapidly proliferating centroblasts could partly explain the significant correlations found in the benign lymphoid tissues between telomerase activity and the fraction of B cells, as well as between telomerase activity and cell proliferation. This could imply a relationship between telomerase expression and cell proliferation in B cells. The finding that mitogen-stimulated B cells in short-term cultures upregulate telomerase further supports the association between telomerase expression and an active cell cycle.23 In preliminary experiments we have detected a similar activation of telomerase in anti-IgM activated B cells at levels that are comparable to our findings in the benign lymphoid tissues.

The mortality stage M1/M2 theory, which is based on analysis of cultured cells, proposes that cells at a critically short telomere length enter a state of crisis and cell death. The rare cells escaping the M2-crisis are immortalized and one prerequisite for immortalization is telomerase activation.11 A telomere length-dependent activation of telomerase is also exhibited by EBV-transformed lymphoblastoid cell lines showing telomerase activation at a stage when telomere reduction is notable.12 In accordance with this one early EBV-transformed lymphoblastoid cell line exhibited very low telomerase activity. This temporal pattern of telomerase reactivation is not exhibited by mitogen stimulated B cells in short-term cultures, which rapidly upregulate telomerase activity. The levels at which telomerase is reactivated in vitro and the even higher levels detected in GC-B cells suggest that a significant fraction of these cells have reactivated telomerase. In the short-term cultures it is improbable that rare cells with preexisting telomerase activity could have proliferated to account for the increase seen in activity. Taken together the data favor the existence of a telomere-length independent activation mechanism of telomerase in normal B cells in vitro and most likely in vivo.

In normal lymph nodes the telomere restriction fragment lengths are >10 kbp (our unpublished data, 1995) and also PB lymphocytes have telomere restriction fragment lengths that are longer than the critical size for telomerase activation.36 The cumulative number of cell divisions for B cells

![Fig 4](image-url)
TELOMERASE ACTIVITY IN LYMPHOID CELLS

Fig 5. Correlation between relative telomerase activity and S-phase fractions in benign lymphoid tissues (benign lymph nodes and benign tonsils) and malignant lymphomas of B-cell derivation. (A) Telomerase activity versus S-phase fraction in benign lymphoid tissues. $r = .82$, $P < .001$. (B) Telomerase activity versus S-phase fraction in malignant lymphomas. $r = .15$, $P = .56$. For information regarding S-phase fractions, see Materials and Methods.

The inability of normal B cells for sustained proliferation in vitro and the subsequent cell death by apoptosis is not a proof of a mortal phenotype. It can be argued that the culture did not provide the microenvironment necessary for the cells to reach their proliferative potential. In immortalization studies based on the M1/M2-in vitro model telomerase reactivation occurs at M2-crisis and is expressed in the rare cells becoming immortalized. Because of the different regulation of telomerase activity in the mitogen stimulated B cells, conclusions concerning the immortalization status of these cells and the GC-B cells would be speculative. Because we are still awaiting a genetic definition of immortalization, the question whether telomerase-positive normal B cells are immortalized remains open.

It seems reasonable to apply the M1/M2-in vitro model for immortalization to the in vivo situation when a tumor after a large number of cell divisions at shortened telomeres reactivates telomerase and to assume that the tumor at this stage contains immortalized cells. The applicability of the M1/M2-model on NHLs that might have retained the telomerase activity found in normal B cells is uncertain. The presence of GC-B cells with a hypothetically indefinite proliferative capacity would not be threatening to the host because the cells are dependent of interactions with other cells (follicular dendritic cells and T cells) for their preservation.

The situation becomes different if one of these cells acquired a tumorigenic phenotype and escaped the stromal dependency. If lymphomas with retained telomerase activity initially are mortal, it seems reasonable to assume that they would have a predisposition to early immortalization. The significant telomerase activity expressed within the B-cell compartment and the fact that most malignant NHLs of non-viral origin predominantly are B-cell derived might support a role for telomerase in tumor development.

All the lymphoma samples exhibited telomerase activity but it cannot be excluded that a few cases exhibiting low levels of telomerase were de facto telomerase-negative due to the possibility of remaining benign background activity. In the high-grade lymphomas significantly increased levels of telomerase compared with the low-grade cases were detected. It is not clear what this stands for in the context of tumor progression. The different levels of telomerase expression between the high- and low-grade lymphomas could be caused by a beginning selective process and reflect the varying demands of telomerase activity to prevent telomere shortening. There might be levels of telomerase activity insufficient to compensate for the telomere loss on each cell-cycle traverse eventually leading to telomere shortening and crisis with cessation of proliferation in a tumor. There was no correlation between telomerase activity and cell proliferation nor between telomerase activity and B-cell fractions in the lymphoma group. One explanation for the lack of correlation is development of clonal heterogeneity within the lymphomas affecting the cell cycle and/or the relation between the cell cycle and telomerase expression. To elucidate if the level of telomerase activity in lymphomas is of importance for tumor progression and whether the seemingly proliferation independent expression of telomerase is caused by deregulation of the enzyme will require future studies.

Two models for telomerase activation during leukemogenesis have been proposed. The "reactivation" model suggests a neoplastic event in a telomerase-negative cell leading to an extended life-span, shortening of the telomeres, and reactivation of telomerase. This model is based on a telomere...
length-dependent reactivation of telomerase while the “expansion and retention” model suggests a neoplastic event in an undifferentiated already telomerase-positive cell. One way to acquire telomerase activity during lymphomagenesis seems to be a retention of the telomerase activity expressed in normal mature B cells. B cells can reactivate telomerase in a telomere length-independent manner by various activation signals, one being immune stimulation exemplified by the germinal center cells. The transformation event leading to a lymphoma can thus occur in a telomerase-positive normal B cell whereas the activity may be retained in the clonal cell population. * Activation signal leading to a reactivation of telomerase activity, which is independent of the telomere length.

It is evident from our data that telomerase can be activated in normal as well as in neoplastic lymphocytes in vivo, and accordingly is unrelated to the neoplastic process per se. The detection of telomerase positivity in tumors with a local immunologic response must therefore be interpreted with caution. Our findings have important implications for future efforts to treat tumors with antitelomerase drugs, and the effects on the immune system of such therapy need to be thoroughly studied.

Fig 6. Acquisition of telomerase activity in malignant lymphomas according to the “induction and retention” model. The model describes one way to acquire telomerase activity in lymphomas. Normal, mature B cells can reactivate telomerase by various activation signals in a telomere length-independent manner. One probable signal is immune stimulation. Transformation to a lymphoma can thus occur in a telomerase-positive normal B cell whereas the activity may be retained in the clonal cell population. * Activation signal leading to a reactivation of telomerase activity, which is independent of the telomere length.

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