Differences in telomerase expression by the CD1a+ cells in Langerhans cell histiocytosis reflect the diverse clinical presentation of the disease

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Abstract

Langerhans cell histiocytosis (LCH) is a disease characterised by an uncontrolled clonal proliferation of Langerhans cells, whose aetiology is still unclear. The clonal nature of LCH could support the hypothesis that it is a neoplastic disease with unlimited growth potential. One requirement for unlimited proliferation is the maintenance of telomere length. In a group of 70 patients we set out to investigate whether a telomere maintenance mechanism is indeed active in LCH cells. This work showed that LCH cells from all restricted skin LCH lesions (6/6) expressed telomerase as assessed by human telomere reverse transcriptase (hTERT) immunohistochemistry, whereas LCH cells from the majority of the bone lesions analysed did not express hTERT (26/34). Interestingly, in contrast to the solitary bone lesions, LCH cells from lesions of multisystem patients always expressed telomerase (11/11), regardless of the lesional site. In situ telomeric repeat amplification protocol (TRAP) assays performed on different lesional sites showed that this telomerase was active. In addition, the telomere length of LCH cells from a hTERT-positive skin multisystem lesion was long and homogeneous when compared to that in the LCH cells from hTERT-negative bone single system LCH lesions, which was heterogeneous in length. No evidence for an alternative lengthening of telomeres mechanism was found in hTERT-negative lesions. The difference in telomerase expression and telomere length at the different lesional sites and in biopsies from patients with solitary versus multisystem disease appears to reflect the diverse clinical presentation and course of this disease. The results from this study have important implications for understanding the nature of this disease.

Introduction

The aetiology of Langerhans cell histiocytosis (LCH), a disease mainly occurring during childhood and characterized by an uncontrolled clonal proliferation of Langerhans cells (LCs) in several sites of the body, is unknown. While some believe that it occurs due to an external trigger, others argue that it is likely caused by an intrinsic defect and is neoplastic (1, 2). Arguments supporting a reactive origin include the granulomatous character of LCH lesions rather than the cellular homogeneity of a malignant neoplasm, frequent cases of spontaneous clinical regression and the failure to establish a cell line from LCH cells in vitro (3). In contrast, observations of several cases of familial clustering, together with studies showing evidence for cytogenetic aberrations confirm that there indeed exists a component of genetic instability in LCH cells (4–7). Furthermore, the clonal nature of LCH could support the hypothesis that this disease is neoplastic (8, 9).

The massive accumulation of LCs that characterizes LCH lesions may result not only from the ab-
normal local proliferation of these cells but also from deregulation of apoptosis. As well as their local proliferative activity, as evidenced by their Ki-67 positivity, LCH cells have been shown to express elevated levels of the anti-apoptotic proteins bcl-2 and survivin (10-12). Furthermore, only a small number of apoptotic cells have been evidenced in LCH lesions by terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) (13), suggesting that, despite the uncontrolled proliferative capacity of LCH cells, they are able to survive. One requirement for such unlimited proliferation is the maintenance of telomere length (14, 15). Telomeres are DNA sequence repeats complexed with specific binding proteins that are located at the ends of every normal human chromosome. They play an important role in chromosome structural integrity and protection against the activation of DNA damage checkpoints. They also counter the loss of terminal DNA segments that occurs when linear DNA is replicated. In normal human cells, telomeres shorten with each cell division and become dysfunctional, leading to chromosomal instability and ultimately cell death (16,17). Therefore, normal human committed cells have a limited proliferative potential. In contrast, pathological telomere elongation is found in a large majority of cancers, either via telomerase enzymatic activity or by an alternative telomerase-independent pathway thought to involve homologous recombination, known as alternative lengthening of telomeres (ALT) (18-20). To date, evidence for ALT activity has only been found in a subset of in vitro immortalized and tumour-derived cell lines, as well as in spontaneous human tumours (21).

The presence of a possible telomere maintenance mechanism in the pathogenic LCs of LCH lesions has not been investigated to date. However, it is clear that the proliferation and survival of LCH cells lies at the heart of the development and persistence of LCH lesions. Therefore, determining how telomeres are regulated in LCH cells has important implications for the nature of this disease.

Methods

LCH tissue samples and controls

Fifty-one paraffin embedded biopsies and 28 frozen biopsies from 70 children with single system (SS) (n = 59) or multisystem (MS) (n = 11) disease were studied, including 39 bone, 15 skin, eight lymph node (LN), nine lung LCH cases and eight cases involving other lesional sites. All received a definite diagnosis of LCH based on morphology and CD1a and S100 immunoreactivity. Dermatopathic lymphadenopathy (DL) (n = 3), was used for comparison. The osteosarcoma cell line (U2OS) was used as a positive control and Ewing’s sarcoma was used as a negative control for the ALT mechanism (22, 23). Giant cell tumour (GCT) and normal testis were used as positive controls for the expression and activity of telomerase, respectively (24, 25). The expression of telomerase by multinucleated giant
cells (MGCs) was also studied in Paget’s disease of bone (n = 3), sarcoidosis (n = 2) and tuberculosis (n = 3). The experiments were approved by the ethics committee of Leiden University Medical Center, and were in accordance with national ethical guidelines (Code of Proper Secondary Use of Human Tissue in The Netherlands, Dutch Federation of Medical Scientific Societies).

**Immunohistochemistry for telomerase**

Paraffin-embedded and frozen sections (4 μm thick) were cut and stained as described previously (26), using a mouse monoclonal anti-hTERT antibody (Novocastra, Newcastle upon Tyne, UK) (27). The primary antibody was detected enzymatically for brightfield microscopy and by immunofluorescence. For enzymatic detection, a rabbit-anti-mouse biotinylated antibody and streptavidin-biotin complex coupled with horse radish peroxidase system (DAKO, Denmark) were used. The colour was developed with 3,3’-diaminobenzidine, the slides were mounted with Pertex and analysed by bright-field microscopy. Double immunofluorescent staining was performed using mouse monoclonsals anti-CD1a (Neomarkers, Fremont, CA, USA) in the case of LCH tissue, or anti-vitronectin receptor (Novocastra) in the case of GCT, and anti-hTERT antibodies, followed by detection with goat anti-mouse Alexa Fluor 488 and 594 (Invitrogen, Carlsbad, CA, USA) and mounting the slides in anti-fading medium. As a negative control, the primary antibodies were replaced by phosphate-buffered saline with 1% bovine serum albumin. For each lesion studied, at least 100 CD1a+ cells were scored for the typical nuclear and/or nucleolar staining of hTERT positivity (27). A complete absence of CD1a+ cells expressing hTERT was considered negative and a positive case scored according to whether it contained 1-30%, 30-70% or 70-100% of hTERT-positive (hTERT+) CD1a+ cells.

**Detection of ALT-associated promyelocytic leukaemia bodies (APBs)**

ALT-APBs were detected in sorted LCH cells from single cell suspensions obtained from paraffin-embedded biopsies (28). Staining was performed using a mouse anti-human Langerin monoclonal antibody (Novocastra) and the secondary antibody goat anti-mouse-488 (Invitrogen). Flow cytometric analysis and sorting were performed using a BD FACSaria (BD Biosciences, San Jose, CA, USA). Controls included isotype-negative control mouse IgG2b from DAKO (Glostrup, Denmark). Langerin/488+ sorted cells were spotted on slides, dried and swollen for 15 min with a borate solution (0.1 M Na₂B₄O₇). Detection of APBs was performed by fluorescent in situ hybridization (FISH) as described (22). The slides were hybridized with a Cy3-labelled telomere-specific peptide nucleic acid (PNA) probe (DAKO), followed by labelling with a goat anti-PML polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA) and detected with an Alexa Fluor 488 labelled secondary antibody. An APB was considered to be present if the telomeric DNA was colocalized with a PML in the nucleus.
An LCH biopsy was considered positive for APBs if present in larger or equal to 20% of the sorted Langerin+ cells. The slides were visualized using a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany) and analysed with Leica QFISH software (Leica Imaging System, Cambridge, UK).

In situ TRAP assay
The telomerase PCR ELISA detection kit from Roche (Basel, Switzerland) was used and adapted for in situ detection of telomerase activity, as described by Youssef et al. (29). This assay enables specific in situ detection of PCR-amplified telomerase-mediated elongation products on frozen sections.

Telomere length determination in sorted CD1a+ cells from LCH and DL frozen biopsies
Frozen LCH and DL biopsy sections, 50 μm thick, were fixed for 1 hr at 4°C with 4% paraformaldehyde. Following mechanical disaggregation, the single cell suspensions were stained with a mouse monoclonal CD1a-APC antibody (BD Pharmingen, San Diego, CA, USA) for 1 hr at 4°C and CD1a+ cells were sorted as above. Sorted cells from the CD1a+ and CD1a-negative (CD1a−) fractions were spotted on glass slides, dried and double stained fluorescently with the mouse anti-CD1a and anti-Langerin monoclonal antibodies, followed by counterstaining with 4’-6-Diamidino-2-phenylindole (DAPI) to confirm the purity of the sorting. The telomere length of the CD1a+ sorted cells was determined using the TeloTAGGG Telomere Length Assay from Roche (Basel, Switzerland), according to the manufacturer’s instructions.

Results

Expression of telomerase by CD1a+ cells in skin but not bone LCH lesions
To investigate whether LCH cells display a telomere maintenance mechanism, the expression of telomerase was studied in LCH lesions from various tissue sites. The staining pattern of the anti-hTERT antibody was confirmed by a control tissue, GCT, where the MGCs express hTERT, the catalytic subunit of telomerase (Figure 1 A). In all the LCH skin lesions studied (n = 15), double immunofluorescent staining of CD1a and hTERT revealed hTERT positivity, not only by the keratinocytes in the epidermis but also by CD1a+ LCH cells in the dermis of LCH skin lesions (Table 1, Figure 1 C). In contrast, LCs in normal control skin were negative for hTERT (Figure 1 D) and only the keratinocytes expressed hTERT (30). The same staining was also performed in SS LN and bone LCH lesions. However, in contrast to SS skin LCH lesions, only 3/5 SS LN lesions and 8/34 SS bone LCH lesions contained CD1a+ LCH cells expressing hTERT (Figures 1 E and F, respectively). Double immun-
of fluorescence for CD1a and hTERT used to study three cases of the reactive disease, DL, showed the LCs to be hTERT-positive (hTERT⁺; data not shown).

Figure 1. Expression of hTERT by CD1a⁺ LCH cells is consistently observed in skin LCH lesions. (A) hTERT staining was confirmed on control tissue known to be positive for telomerase, giant cell tumour (GCT). Double immunofluorescent staining was performed using the vitronectin receptor (green) to identify the multinucleated giant cells in GCT, in combination with hTERT (red) and DAPI (blue). (B) The omission of the primary antibodies in GCT confirmed the specificity of this staining. (C) Double immunofluorescence staining was performed using CD1a as a LCH cell marker (green) in combination with hTERT (red). DAPI was used to distinguish the cell nuclei (blue). Besides the keratinocytes in the epidermis (e), hTERT was also expressed by CD1a⁺ LCH cells in the dermis (d) of skin LCH lesions (see inset picture), in contrast to (D) normal skin, where the LCs were negative for hTERT. (E, F) Representative examples of LN and bone LCH lesions in which the CD1a⁺ cells were negative for hTERT in the majority of cases. The broken white line depicts the epidermal-dermal junction in the skin (magnifications: A, B, 40x; C, D, E, F, 100x).
Table 1. Detection of telomere maintenance mechanisms in LCH lesions.

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>hTERT+ CD1a cells§</th>
<th>hTERT+ MGCs*</th>
<th>Telomerase activity†</th>
<th>ALT‡</th>
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<tr>
<td></td>
<td>1-30% 30-70% 70-100%</td>
<td>4-30% 30-70% 70-100%</td>
<td></td>
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<tr>
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<td>5/11: 2 1 2</td>
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<td>2/2: 1 1 0</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>SS other lesional site</td>
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<td>3/3: 1 1 1</td>
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§Number of lesions out of the total studied which contain CD1a+ cells expressing hTERT. In each of the cases of an hTERT+ lesion, the percentage of CD1a+ cells expressing hTERT was scored into three groups: 1-30%, 30-70% or 70-100%.

*Number of lesions out of the total studied which contain MGCs expressing hTERT. In each of the cases of an hTERT+ lesion, the percentage of MGCs expressing hTERT was scored into three groups: 1-30%, 30-70% or 70-100%.

†Number of lesions out of the total studied which contain CD1a+ cells displaying telomerase activity.

‡Number of lesions out of the total studied which contain CD1a+ cells displaying an alternative lengthening of telomere (ALT) mechanism.

Multinucleated giant cells in skin LCH lesions also express hTERT

Another cell type intrinsic to LCH lesions is the MGC. The expression of hTERT by these cells was also investigated by single enzymatic staining. In addition to the CD1a+ LCH cells displaying hTERT positivity, the MGCs also expressed hTERT in the skin (Figure 2 A), LN and a few cases of bone LCH lesions that contained MGCs (Figure 2 B). This was observed in nine cases of LCH, of which five were LCH lesions that contained hTERT+ LCH cells. These findings were compared to other diseases that contain activated MGCs, such as sarcoidosis and Paget’s disease. In these diseases MGCs were also positive for hTERT (Figures 2 C and D, respectively).

Telomerase detection in bone LCH lesions from multisystem patients

As well as lesions from SS disease patients, the CD1a+ LCH cells of different lesional sites from 11 patients with MS LCH were also studied for their expression of telomerase. In 11 out of 11 MS patients, hTERT was expressed by the LCH cells regardless of the lesional site. Even the LCH cells from bone lesional sites showed hTERT positivity, in contrast to most SS bone LCH lesions. Figures 3 A, B and C show a representative example of a MS LCH patient with skin, bone and gut involvement, respectively. In all these sites LCH cells were hTERT+.

In situ detection of telomerase activity in skin LCH lesions

Although hTERT is a major component of telomerase, its expression does not strictly correlate with telomerase activity. Therefore, the activity of telomerase in hTERT+ LCH biopsies was assessed using an in situ telomeric repeat amplification protocol (TRAP) assay which allows direct visualiza-
tion of telomerase activity on tissue sections. Testis, which contains many germ cells known to have telomerase activity, was used as positive control (Figures 4 A and B). To check the specificity of the technique, negative controls which included the omission of the reaction mixture were routinely used (Figures 4 A, C and E). Telomerase activity was observed in cells present in the dermis of two hTERT+ skin LCH lesions (Figures 4 D and Table 1). In contrast, SS bone LCH lesions which were negative for hTERT (hTERT) did not show any evidence for telomerase activity (Figure 4 F) From the five SS bone cases analysed, only one showed telomerase activity, which corresponded to a lesion containing CD1a+ hTERT+ cells (Table 1).

Figure 2. Expression of hTERT by MGCs in LCH lesions compared to other diseases that typically contain these cells. Immunohistochemistry was performed with an anti-hTERT monoclonal antibody and DAB detection. hTERT was detected in the osteoclast-like MGCs of skin (A) and a few bone LCH lesions (B) (arrows). The expression of hTERT in sarcoidosis (C) and Paget’s disease of bone (D) also showed positivity of the MGCs (arrows) (magnifications: A, B, C, D, 40x).

Lack of an ALT mechanism in hTERT-negative lesions
To determine whether the hTERT LCH cells in bone and LN lesions were displaying an ALT mechanism, the presence of APBs in these cells was determined. This was performed by FACS-sorting the LCH cells using
the LC-marker langerin (Figure 5 A, right panel) and carrying out FISH on the sorted LCH cells, using a PNA probe in combination with fluorescence immunolabelling for PML. The osteosarcoma cell line U2OS was used as a positive control for ALT positivity (Figure 5 B, left panel). hTERT+ LCH cells from SS bone LCH lesions did not show any co-localization of the PNA probe and PML protein (Figure 5 B, right panel and Table 1), demonstrating the lack of an ALT mechanism. In addition, the skin and LN LCH lesions where LCH cells were hTERT+ (15 out of 15 and 6 out of 8, respectively) did not show any co-localization of PML/PNA (data not shown).

**Figure 3.** hTERT expression by CD1a+ LCH cells of a patient with multisystem disease, affecting skin, bone and gut. Double immunofluorescence staining using CD1a (green) in combination with hTERT-specific antibodies (red) was performed in skin (A), bone (B), and gut lesions (C) of a representative multisystem LCH patient. DAPI was used to distinguish cell nuclei. The broken white lines depicts the epidermal-dermal junction in the skin (A) and the crypts of the gut (C) (magnifications: A, B, C: 63x).

**Determination of telomere length in sorted CD1a+ LCH cells**

To evaluate the telomere length of LCH cells, the telomere repeat fragment (TRF) southern blot method was performed with DNA from sorted LCH cells of four LCH biopsies. The specificity of the CD1a staining (Figure 6 A, right panel) was confirmed using an IgG1-APC control (Figure 6 A, left panel). The purity of the sorting was confirmed by spotting the CD1a+ and CD1a- sorted fractions on glass slides and restaining them with CD1a and Langerin antibodies. Only the CD1a+ fraction stained for these LC-specific markers (Figure 6 B). Due to the great difficulty in obtaining frozen LCH biopsies, as well as isolating enough LCH cells from tissues to obtain sufficient amounts of DNA from these sorted cells, we were only able to include one hTERT+ biopsy (skin MS LCH) and three hTERT- biopsies (all SS bone LCH) in this experiment. Nevertheless, a difference between the telomere length of hTERT+ and hTERT LCH cells was observed. Whereas hTERT+ LCH cells from the MS biopsy showed a very homogeneous long telomere length (mean TRF 9.9 Kbp), hTERT cells from the three SS biopsies displayed a shorter (mean TRFs 5.9, 6.7 and 5.7 Kbp) and much more heterogeneous telomere length (Figure 6 C). The same analysis performed on sorted CD1a+ cells from two hTERT+ DL biopsies, showed the LCs from this reactive condition to display a very heterogeneous telomere length (Figure 6 C).
Figure 4. *In situ* detection of telomerase activity in hTERT+ LCH lesions. The activity of telomerase in positive control tissue, testis, and hTERT+ and negative LCH biopsies (B, D and F, respectively) was assessed using an *in situ* telomeric repeat amplification protocol (TRAP) assay. The omission of the reaction mixture confirmed the specificity of the TRAP detection (A, C and E). Only the hTERT+ and not the hTERT- LCH biopsies showed cells positive for TRAP, as demonstrated by representative pictures of (D) a hTERT+ LCH MS skin lesion and (F) a hTERT- LCH SS bone lesion. The broken white line depicts the epidermal-dermal junction in the skin and the arrows and inset pictures demonstrate the TRAP positivity of the cells (green spots) (magnifications: A, B, C, D, E, F, 63x).
Discussion

LCH is a rare disease whose aetiology remains unknown. The clonal, proliferative nature of LCH cells, together with the higher incidence of secondary neoplasms, familial clustering and reports of loss of heterozygosity all contribute to the hypothesis that LCH is a neoplastic disease (4, 5, 8, 9). As a characteristic of neoplastic cells is their ability to survive through the maintenance of their telomere length, we hypothesized that LCH cells are able to activate a telomere maintenance mechanism, resulting in stabilization of telomere length and immortalization.

This study showed that the lesional CD1a+ cells express telomerase in all SS skin LCH lesions, in
Figure 6. Long, homogeneous telomere length of a hTERT+ LCH lesion in contrast to a heterogeneous telomere length of hTERT- LCH lesions. CD1a+ LCH cells were FACS-sorted from frozen biopsies (A, right panel) and the specificity of the staining was confirmed by using an IgG1-APC control (A, left panel). (B) The purity of the CD1a+ and CD1a- sorted fractions was performed by restaining the cells with Langerin and CD1a. (C) Southern blotting of the TRFs was carried out, using DNA from CD1a+ cells from the affected skin of a multisystem (MS) LCH patient, three single system (SS) LCH bone lesions and two DL biopsies. The controls included high (hmw) and low (lmw) molecular weight DNA supplied with the kit. The CD1a+ cells from the MS patient displayed a long homogeneous telomere length, in contrast to the more heterogeneous length displayed by CD1a+ cells from the three SS bone LCH lesions and two DL biopsies (C). The telomere length (Kbp) is depicted at the bottom of each lane.
activity, as shown by the TRAP assay, suggests that hTERT expression is indeed a reliable marker of telomerase activity. Furthermore, we determined the telomere length of LCH cells from an hTERT+ MS skin lesion, compared to three SS bone LCH lesions that were hTERT-. Although the number of biopsies studied was small, our results showed a difference in telomere length between the hTERT+ lesion and the three hTERT- lesions. Whereas the telomere length of LCH cells in the MS skin lesion was homogeneous and long, the telomere length of LCH cells in the three hTERT- SS LCH lesions was heterogeneous. This difference in telomere length and telomerase expression in the different lesional sites and forms of the disease may reflect the broad clinical spectrum of LCH, which ranges from a lethal leukaemia-like disorder, in which multiple organs are involved, to a curable solitary lytic lesion of bone. Indeed, in our patient cohort, telomerase was consistently expressed by LCH cells in MS disease and appeared to result in elongated telomeres. However, this correlation should be further investigated, as the hTERT positivity of LCs in the reactive condition, DL, did not appear to correlate with increased telomere length. In contrast, as the loss of telomerase activity has been correlated with cell senescence (31), the finding that the majority of SS bone lesions were negative for any telomere maintenance mechanism suggests that the LCH cells in these lesions may indeed have a more limited proliferative capacity and life-span. This could explain the fact that many patients with SS disease affecting the bone require minimal treatment or the lesions resolve spontaneously and hardly ever recur.

The lack of telomerase expression by LCH cells in the majority of SS bone lesions compared to skin lesions, which were always positive for this enzyme, was quite striking. One possible explanation for this difference could be due to the lesional environment, as different cytokines have been shown to have a profound impact on the regulation of telomerase (32). Although telomerase expression is mainly associated with cancers and immortalized cell lines, and is therefore thought to be involved in malignant transformation and cellular immortality (33), telomerase activity can also be detected at low levels in normal bone marrow and peripheral blood lymphocytes and at higher levels in activated T and B cells (34, 35). To date, there are only very limited data on the telomere length and telomerase activity in myeloid-lineage cells. In our study, the pathological LCs in skin LCH lesions expressed telomerase, in contrast to normal LCs from unaffected skin, which were negative for telomerase. Importantly, the LCs from the reactive condition DL were positive for telomerase, as they were in the majority of lung LCH lesions which is thought to be a more reactive disease, associated with cigarette smoking (36). Furthermore, although the MGCs in some LCH lesions were telomerase-positive, this was also the case for the MGCs found in the non-neoplastic conditions GCT, Paget’s disease, sarcoidosis and tuberculosis. Thus, the expression of telomerase does not necessarily distinguish malignant proliferations from reactive states. The detection of telomerase positivity in LCH lesions, where there is a clear immunological response occurring, must therefore be interpreted with caution.
Telomerase activation may instead serve to protect cellular, proliferative capacity and to prevent apoptosis, hence potentiating LC survival in LCH lesions.

This study shows for the first time a clear difference between SS skin and bone LCH lesions and SS versus MS disease, based on the expression of telomerase. This difference in telomerase expression appears to be reflective of the diverse clinical presentation and course of the disease. However, the measurement of telomere length of LCH cells in a larger cohort of LCH patients is needed to validate our findings and will help to increase our understanding of the nature of the various clinical forms of this disease.

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