

ORIGINAL ARTICLE

Enhanced telomere shortening in transformed lymphoblasts from patients with X linked dyskeratosis

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Aim: Dyskeratosis congenita (DC) is characterised by the failure of those tissues that are rapidly dividing in the adult, particularly the skin, mucosae, and haemopoietic system. The X linked form of the disease is caused by mutations of the DKC1 gene, which encodes dyskerin, a protein that is necessary for the function of telomerase. Cultured DC lymphoblastoid cells are characterised by a reduced expansion of the cell population because of the progressive increase in apoptosis compared with the number of cell divisions. This report aimed to verify whether this is caused by a defect in telomerase function.

Methods: Variations in telomere length over time were evaluated in two cultured lymphoblastoid cell lines derived from patients with X linked DC and control cells derived from a non-affected individual. In addition, the effect of inhibiting poly (ADP-ribose) polymerase (PARP), which is involved in the cellular response to excessive telomere shortening, was assessed. One DC cell line and the control cells were treated with the specific PARP inhibitor 1,5-dihydroxyquinoline (IQ).

Results: In DC cells the increase in cell death was associated with progressive telomere shortening, and this was not seen in the control cells. Treatment with IQ delayed the increase of apoptosis in DC cells.

Conclusions: These observations indicate that the reduced expansion that characterises cultured cells obtained from patients with X linked DC is caused by premature telomere shortening.

X linked dyskeratosis congenita (DC; OMIM 305000) is a rare inherited disorder characterised by the triad of reticulate skin pigmentation, nail dystrophy, and mucosal leucoplakia. In addition, several non-cutaneous abnormalities are present, the most clinically relevant of which is the progressive bone marrow failure that occurs in over 80% of patients.^{1,2}

X linked DC is caused by mutation of the DKC1 gene,^{3,4} which encodes dyskerin, a protein that localises to the nucleolus.⁵ Heiss and co-workers suggested that the proliferative defects described in DC could be ascribed to altered ribosome biogenesis because dyskerin is a homologue of the yeast and rat rRNA pseudouridine synthases.³ Moreover, in a recent paper describing a mouse model of DC in which the expression of the DKC1 gene was reduced, the clinical features typical of DC appeared to be associated with defects in rRNA pseudouridylation.⁶ Alterations in dyskerin have also been described in other pathological entities; indeed, different mutations of the DKC1 gene can lead to the Hoyeraal-Hreidarsson syndrome (OMIM 300240), which is characterised by very early symptoms (growth retardation of prenatal onset, microcephaly, cerebellar hypoplasia, pancytopenia, and death at an early age).⁷

"A causative role for a defect in telomerase in dyskeratosis congenita (DC) is also supported by the observation that the more rare autosomal dominant form of DC is caused by mutations of the RNA component of the telomerase complex"

In a previous study performed in transformed lymphoblasts from patients with DC, we detected no defects in rRNA transcription and maturation and found that the proliferative capability of the cells is not affected. Moreover, we demonstrated that X linked DC cell lines are characterised by a reduced expansion of the cell population because of the strong and progressive increase in the proportion of apoptotic cells

over time.⁸ Such observations are consistent with the data of Mitchell *et al*, who reported the presence in DC cells of a telomerase malfunction, which might be responsible for the progressive defects of proliferating tissues.⁹ A causative role for a defect in telomerase in DC is also supported by the observation that the more rare autosomal dominant form of DC is caused by mutations of the RNA component of the telomerase complex.¹⁰ In proliferating cells, a defect in the function of telomerase causes premature telomere shortening and leads, depending on the cell type, either to proliferative senescence or to apoptosis.^{11–13} Taken together, these data suggest that in DC lymphoblasts the progressive increase in the number of apoptotic cells is caused by progressive telomere shortening as a result of altered telomerase function.

The aim of our study was to verify whether, in continuously proliferating cells derived from patients affected by X linked DC, the progressive increase of cell death is caused by progressive telomere shortening. For this purpose, we first evaluated the variation in telomere length over time in cultured lymphoblastoid cell lines derived from patients with DC and in control cells derived from a non-affected individual. In X linked DC cells, the increase in cell death turned out to be associated with progressive telomere shortening, which was not seen in control cells. Second, to verify whether the increase in apoptosis was caused by telomere shortening, we evaluated the effect of inhibiting poly (ADP-ribose) polymerase (PARP), an enzyme involved in the cellular response to excessive telomere shortening.^{11–13} For this purpose, we treated one DC cell line and the control cells with 1,5-dihydroxyquinoline (IQ), a specific PARP inhibitor.¹⁴ This treatment delayed the increase of apoptosis in DC cells.

Abbreviations: DC, dyskeratosis congenita; EBV, Epstein-Barr virus; FITC, fluorescein isothiocyanate; IQ, 1,5-dihydroxyquinoline; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; PNA, peptide nucleic acid; RTL, relative telomere length

Table 1 Cell death and proliferation in the cell lines studied over a period of 4 weeks

Cell lines	Percentage of dead cells (mean (SD))			
	1st week	2nd week	3rd week	4th week
GM00130c	16 (5)	18 (7)	19 (4)	25 (3)
GM00130c+IQ		16 (4)	17 (9)	30 (3)
GM03193	19 (4)	35 (7)	45 (11)	69 (8)
GM03194	17 (3)	14 (4)	70 (4)	89 (11)
GM03194+IQ		17 (1)	23 (7)	62 (13)

Cell lines	Cytofluorimetric hypodiploid peak (%)			
	1st week	2nd week	3rd week	4th week
GM00130c	5.6	4.9	4.2	5.3
GM00130c+IQ		6.9	5.2	5.6
GM03193	8.2	16.8	20.2	48.7
GM03194	12.7	17.9	47.6	95.6
GM03194+IQ		17.0	22.8	78.0

IQ, 1,5-dihydroxyquinoline.

MATERIALS AND METHODS

Cell cultures

Four cell lines (GM01775, GM03193, GM03194, and GM00130c), derived from lymphocytes immortalised by Epstein-Barr virus (EBV), were obtained from Coriell Cell Repositories (Coriell Institute for Medical Research, Camden, New Jersey, USA). The GM03193 and GM03194 cell lines derive from two brothers affected by X linked DC and harbour an A → G missense mutation in the DKC1 gene transcript at position 196 (T66A).³ The GM01775 cell line, to our knowledge the only DC lymphoblastic cell line from a different family available in public repositories, harbours a CTT deletion at position 201–203, which leads to a single amino acid deletion (L37).⁴ The number of culture passages before the investigation was not available for the GM01775 and GM03193 cell lines and was zero for GM03194. The control GM00130c cell line derives from a non-affected unrelated man. The age of the patients at the time of culture establishment was 7, 15, 18, and 25 years for GM01775, GM03193, GM03195, and GM00130c, respectively. The human neoplastic haemopoietic 1301 cell line,¹⁵ used as a telomere length internal control in cytofluorimetric determinations of telomere length, was purchased from Interlab Cell Line Collection (Genoa, Italy).

The cells were grown as a suspension culture in RPMI 1640 containing 20% heat inactivated fetal bovine serum under standard conditions, and were maintained at a concentration of approximately 200 000 viable cells/ml by adding fresh medium to the culture.

Cells were cultivated for three days immediately after their arrival from the Coriell Cell Repository. Then, for each cell line, several aliquots of cells were stored in liquid nitrogen. For the weekly evaluations, an aliquot of each cell line was thawed and cultured as described above. Each week, the cells needed for mortality and proliferation rate evaluations were taken from each culture. To inhibit PARP activity, cultures were supplemented with 100 μM IQ (Sigma-Aldrich, Milan, Italy). This concentration of IQ has been shown to give the greatest extension of life span when added to senescent fibroblast cultures.¹¹

Cytofluorimetric analysis of telomere length

The determination of telomere length was performed by fluorescence in situ hybridisation and flow cytometry analysis.^{16, 17} This method was preferred to the determination of telomere restriction fragment length by Southern blot analysis, because it requires fewer cells and their reduced availability is the major limitation when working with cells derived from patients with DC. The analysis was performed using the

telomere peptide nucleic acid (PNA) kit/fluorescein isothiocyanate (FITC) for flow cytometry (DAKO A/S, Glostrup, Denmark). Four aliquots of 2×10^6 live cells from the GM00130c, GM03193, and GM03194 cell lines were harvested during the first and the third week of culture and frozen in liquid nitrogen with 10% DMSO. Then each aliquot was thawed, mixed with 2×10^6 cells from the 1301 cell line, and washed twice in phosphate buffered saline. The 1301 cell line is used as an internal control because it is tetraploid and has very long telomeres (> 30 kb), and can therefore be distinguished from the cell types used in the assay.¹⁶ After centrifugation, samples were denatured, hybridised, and stringently washed according to the manufacturer's protocol and using the solutions provided with the kit. For each measure, a duplicate analysis (hybridisation with telomere PNA probe) and a duplicate control (hybridisation without telomere PNA probe) were performed. After washing, samples were analysed on a Coulter Epics XL (Coulter Corp, Miami, Florida, USA) flow cytometer using the logarithmic scale FL1-H for probe fluorescence and the linear scale FL3-H for DNA staining. Gates were set around the G0/G1 phase for both lymphoblasts and control cells based on DNA content. Relative telomere lengths (RTLs) were then determined in the following way, according to the manufacturer's protocol:

$$(\text{mean FL1 lymphoblast cells with probe} - \text{mean FL1 lymphoblast cells without probe}) \times \text{DNA index of 1301 cells} / (\text{mean FL1 1301 cells with probe} - \text{mean FL1 1301 cells without probe}) \times \text{DNA index of lymphoblast cells}$$

Cytofluorimetric analysis of apoptotic fraction

Cells were collected by centrifugation, fixed in 70% ethanol, and stained with propidium iodide (PI) using the DNA-PREP stain kit (Coulter Corp), according to the manufacturer's instructions. The analysis was performed using a Coulter Epics XL (Coulter Corp). Apoptotic cells were identified and measured on the PI fluorescence histogram as a hypodiploid peak, as described previously.¹⁸ Each sample was analysed in duplicate and the mean of the two values taken.

RESULTS

GM01775 cells could not be used for experimental evaluations because of a sudden increase in cell mortality, which resulted in insufficient numbers of cells.

Continuously proliferating transformed lymphoblastoid cell lines GM00130c (control), GM03193, and GM03194 (both X linked DC) were maintained in culture for four weeks. At the end of each week, cell viability was determined both by the trypan blue exclusion test and by the evaluation of the

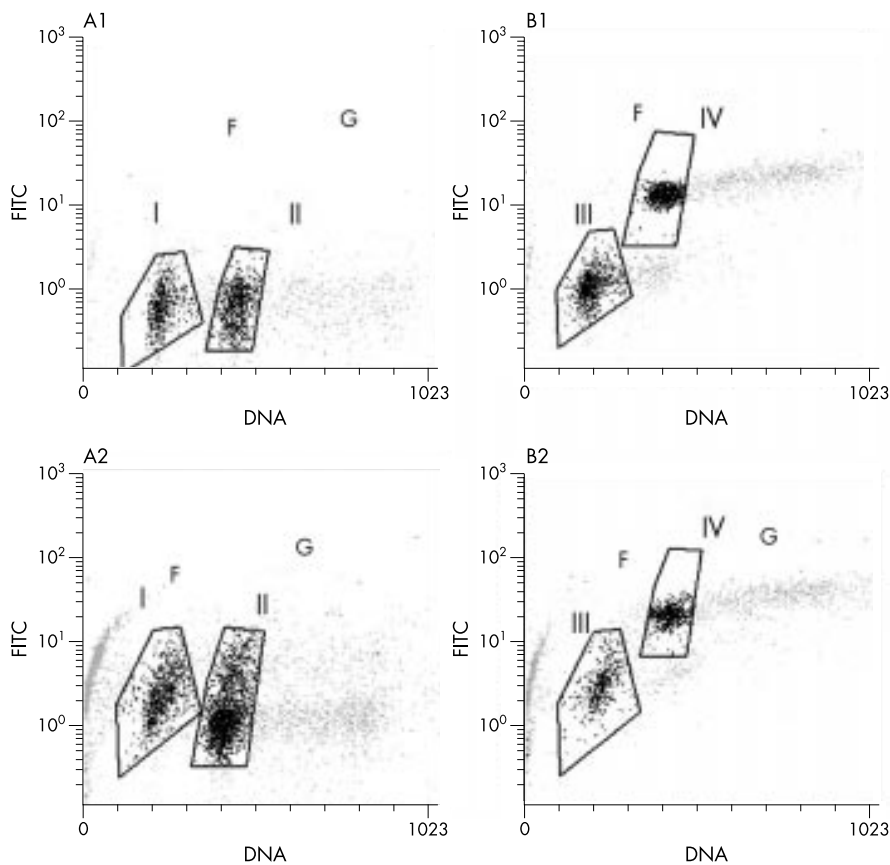


Figure 1 Cytofluorimetric analysis for the determination of relative telomere length (RTL) in the GM03194 DC lymphoblastoid cell line at the first (A1, B1) and at the third (A2, B2) weeks of culture. In (A1) and (A2), GM03194 (I) and 1301 (II) tetraploid internal control cells were stained with propidium iodide (PI); in (B1) and (B2), GM03194 (III) and 1301 (IV) cells were in situ hybridised with a telomere specific fluorescein isothiocyanate labelled peptide nucleic acid probe and stained with PI. The mean RTL of the GM03194 cells during the first week of culture was 4.7% (SD, 0.9%) of the telomere length of the 1301 internal control cells, whereas at the third week it was 2.8% (SD, 1.1%).

hypodiploid peak after PI staining and flow cytometry. Using both methods, the results confirmed that DC GM03193 and GM03194 are characterised by a progressive and steep increase in mortality over time, whereas cell mortality remains constantly low in the control (table 1).

Telomere length was evaluated in DC lymphoblasts GM03193 and GM03194 and in control cells GM00130c by cytofluorimetric analysis using a telomere specific FITC conjugated PNA probe. The values obtained were standardised with those of the internal control of the analysis, represented by the 1301 leukaemic cell line characterised by a stable telomere length. In this way, RTL was calculated and expressed as a percentage of the length of the 1301 cells. In GM00130c control cells, the telomere length remained quite constant, ranging from a mean RTL of 10.1% (SD, 5.7%) in the first week to a mean RTL of 10.6% (SD, 2.6%) in the third week (not significant). In contrast, in the DC cell lines GM03193 and GM03194, telomere length was significantly reduced. Indeed, the mean RTL changed in the GM03193 cell line from 8.4% (SD, 0.7%) in the first week to 2.7% (SD, 1.7%) in the third week ($p < 0.01$), and in GM03194 from 4.7% (SD, 0.9%) in the first week to 2.8% (SD, 1.1%) in the third week ($p = 0.035$) (fig 1). Analysis was not performed after the third week of culture because of the excessively low number of live cells in the DC cultures.

With the aim of verifying whether telomere shortening was responsible for the progressive increase of cell death in X linked DC cell lines, we evaluated the effect of PARP inhibition on cell mortality by treating the GM00130c (control) and GM03194 (DC cells) with 100 μ M IQ. We saw a delay in the cell mortality increase in DC cells, whereas there was no effect in the control cell line (table 1). By the third week of culture, the mean RTL of the GM03194 cells cultured with IQ was 2.6 (SD, 1.2%), which was not significantly different from the value obtained in the same cells cultured without IQ (mean, 2.8; SD, 1.1%; NS).

DISCUSSION

In our study, we demonstrate significant telomere shortening in two continuously proliferating lymphoblastoid cell lines derived from patients affected by X linked DC. This telomere shortening was associated with a progressive increase of the apoptotic cell fraction. Such an increase in cell mortality was delayed by treating the cells with IQ.

Data reported in the literature indicate that the mutation of dyskerin destabilises the telomerase enzymatic complex, causing it to malfunction.⁹ The presence of very short telomeres has been described both in lymphoblasts (including the cells used for our study) and fibroblasts cultured in vitro,⁹ and in the peripheral blood cells of patients.¹⁹ In our study, for the first time, we demonstrate that in two lymphoblastoid cell lines derived from patients with X linked DC, this change in the length of the telomeres is caused by a rapid and progressive telomere shortening, which was absent in the control line (the mean RTL in the GM03193 cells was reduced from 8.4% (SD, 0.7%) in the first week to 2.7% (SD, 1.7%) in the third week ($p < 0.01$); in GM03194 cells the mean RTL was 4.7% (SD, 0.9%) in the first week and of 2.8% (SD, 1.1%) in the third week ($p = 0.035$)).

"Because poly (ADP-ribose) polymerase activation is involved in the processes that follow telomere erosion, leading either to proliferative senescence or to apoptosis, our data suggest that the increased apoptosis seen in dyskeratosis congenita cells is secondary to premature telomere shortening"

To clarify the relation between telomere shortening and the increase in cell death we studied the effect of PARP inhibition in both the GM03194 DC cell line and in the GM00130c control cell line. Our results indicate that treatment with IQ, a specific PARP inhibitor, delays the increase of apoptosis in the

Take home messages

- The increase in cell death seen in dyskeratosis congenita (DC) cells was associated with progressive telomere shortening, which was not seen in the control cells
- Treatment with the poly (ADP-ribose) polymerase inhibitor 1,5-dihydroxyquinoline delayed the increase of apoptosis in DC cells
- Taken together, these observations indicate that the reduced expansion that characterises cultured cells obtained from patients with X linked DC is caused by premature telomere shortening

DC cell line. This is seen mainly in the third and the fourth weeks of culture (when significant telomere shortening takes place), whereas it had no effect before the third week or in the control cells during all four weeks of the experiment. Because PARP activation is involved in the processes that follow telomere erosion, leading either to proliferative senescence or to apoptosis,^{11,13} our data suggest that the increased apoptosis seen in DC cells is secondary to premature telomere shortening. We also wondered whether treatment with IQ might delay apoptosis via a direct effect on cell proliferation that would retard telomere shortening. However, our data on telomere length in DC GM03194 cells cultured with or without IQ indicate that this was not the case, because IQ has no effect on the telomere shortening rate (the mean RTL in the GM03194 cells was 4.7% (SD, 0.9%) in the first week and 2.8% (SD, 1.1%) in the third week in the absence of IQ and it was 4.7% (SD, 0.9%) in the first week and 2.6% (SD, 1.2%) in the third week in the presence of IQ).

Therefore, our data demonstrate that the progressive increase of the apoptotic cell fraction seen in DC lymphoblasts results from strong and premature telomere shortening. The use of immortalised cells to characterise a disorder involving premature telomere attrition in labile tissues might be questioned. Indeed, telomere erosion in proliferating non-transformed tissues should cause cell proliferative senescence and not apoptosis, which is expected in transformed cells, such as EBV infected lymphoblasts.^{12,13} However, the extremely low prevalence of the disorder and the difficulties in cultivating X linked DC derived cells limited our choice to the cell lines used here. We tried to perform similar experiments with some DC fibroblast cultures available on public repositories, but we could not obtain the necessary number of cells. Despite these limitations, our findings add a new element to the definition of the molecular mechanisms of X linked DC that could explain the increasing severity of the defects of proliferating tissues in relation to the increase of cell cycles observed in DC.^{1,2}

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