Telomerase activity, apoptosis and cell cycle progression in ataxia telangiectasia lymphocytes expressing TCL1

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Individuals affected by ataxia telangiectasia (AT) have a marked susceptibility to cancer. Ataxia telangiectasia cells, in addition to defects in cell cycle checkpoints, show dysfunction of apoptosis and of telomeres, which are both thought to have a role in the progression of malignancy. In 1–5% of patients with AT, clonal expansion of T lymphocytes carrying t(14;14) chromosomal translocation, deregulating TCL1 gene(s), has been described. While it is known that these cells can progress with time to a frank leukaemia, the molecular pathway leading to tumorigenesis has not yet been fully investigated. In this study, we compared AT clonal cells, representing 88% of the entire T lymphocytes (AT94-1) and expressing TCL1 oncogene (ATM− TCL1+), cell cycle progression to T lymphocytes of AT patients without TCL1 expression (ATM− TCL1−) by analysing their spontaneous apoptosis rate, spontaneous telomerase activity and telomere instability. We show that in ATM− TCL1+ lymphocytes, apoptosis rate and cell cycle progression are restored back to a rate comparable with that observed in normal lymphocytes while telomere dysfunction is maintained.

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Ataxia telangiectasia (AT) is an autosomal recessive disease characterised by progressive cerebellar degeneration, variable immunodeficiency, genomic instability and susceptibility to cancer, especially lymphoid malignancies. The product of ATM gene, which is mutated in individuals affected by AT, belongs to a well-conserved family of protein kinases. It is involved in the control of the cell cycle, in the processing of DNA damage and in the maintenance of genome integrity (caretaker gene) (Shiloh and Kastan, 2001). In particular, the Atm protein is homologous to Tel1p, which is essential for telomere maintenance in Saccharomyces cerevisiae (Greenwell et al, 1995; Pandita, 2002), and may regulate the structure and the function of telomeres through a not yet fully understood mechanism; indeed, AT cells have an accelerated rate of telomere loss and show chromosomal end-to-end telomeric fusions/associations (tas), in spite of having normal telomerase activity (Pandita et al, 1995; Metcalf et al, 1996). Furthermore, Atm might play a role in apoptosis as suggested by its involvement as a substrate of caspase 3 (Smith et al, 1999); however, different cell types that lack the ATM function show apoptotic behaviour not always consistent (Meyn, 1999; Bebb et al, 2001).

Tumours of the lymphoid system are very frequent in AT patients. Among them T-prolymphocytic leukaemia (T-PLL), a very rare lymphoid neoplasia in general population, is also quite often observed in AT patients (Taylor et al, 1996). These T-PLLs in ATs have been well characterised in the past by several groups including us, and are quite invariably preceded by a T cell clonal expansion identifiable cytogenetically to carry a t(14;14) (q11;q32.1) translocation or an inv(14)(q11;q32.1) inversion or a t(X;14)(q28;q32.1) translocation. With the time, usually years, these clonal cells are able to expand until they represent up to 90% of the circulating T lymphocytes and ultimately to evolve into an overt leukaemia. These chromosomal rearrangements, at molecular level, bring regulatory elements of the TCRz (located on1q41) to a new position on 14q32.1 or Xq28 resulting in activation of usually silent genes in normal mature T cells. These genes belong to the TCL1 gene family: TCL1 and TCL1b are located on 14q32.1 and MTCPI is on Xq28 (Stern et al, 1993; Virgilio et al, 1994; Pekarsky et al, 1999). Tcl1, Tcl1b and Mtcp1 identify a new family of proteins since they are practically identical, either by sequence or structure, and are usually absent in mature normal circulating T cells. The consequences of TCL1 expression are due to its binding to the serine/threonine kinase Akt (also called protein kinase B, PKB), by increasing kinase activity and enhancing Akt nuclear translocation (Laine et al, 2000; Pekarsky et al, 2000). Furthermore, TCL1 family gene members have oncogenic ability, as clearly demonstrated by four different transgenic animal models in which overexpression of these genes, in either T or B cells, causes T-PLL, B-chronic lymphocytic leukaemia (B-CLL) or mature B-cell lymphoma (Gritt et al, 1998; Virgilio et al, 1998; Bichi et al, 2002; Hoyer et al, 2002).

We previously described a large nonleukaemic clone carrying the chromosomal tandem translocation t(14;14)(q11;q32) in an
AT patient (AT94-1), who, at present, has not yet shown any sign of malignancy. The clonal cells are characterised by the overexpression of the TCL1 oncogene (Narducci et al., 1995), a high rate of spontaneous chromosome instability (especially of tas) (Petrinelli et al., 2001) and hypersensitivity to the topoisomerase II inhibitor VP16 (Petrinelli et al., 1996). These clonal cells represent a very useful tool to study the biological consequences of TCL1 overexpression in the context of ATM deficiency since they are not hampered by ectopic transfection. In this study, we report that in this AT cell clone, even when VP16-damaged, the apoptosis rate and the cell cycle progression are restored back to a rate comparable with that observed in normal lymphocytes while telomere dysfunction is maintained.

MATERIALS AND METHODS

Lymphocyte cultures

Peripheral blood samples were obtained from the AT94-1 patient, from six normal controls and from two AT controls (AT95-1 and AT95-2). AT94-1 and AT95-2 were reported as AT22RM and AT28RM, respectively, by Giald et al (1996).

To evaluate the spontaneous rate of tas, chromosome preparations were performed by standard method, and analysed on G-banded metaphases by two independent observers.

To evaluate telomerase activity, telomere length, cell cycle progression and apoptosis, blood was defibrinated and mononuclear cells isolated by centrifugation on a layer of histopaque 1077 (Sigma Diagnostic, Milan, Italy). Purified lymphocytes (1 x 10^6 ml^-1) were incubated for 72h in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum, penicillin, streptomycin and PHA (5 µg ml^-1). To evaluate the effects of the topoisomerases II inhibitor VP16 on cell cycle and apoptosis, VP16 (final concentration 0.1 µg ml^-1) was added to purified lymphocyte cultures 48h before harvesting (Petrinelli et al., 1996).

Telomerase activity and telomere length

Telomerase activity was measured with the PCR-based TRAP (telomeric repeat amplification protocol) kit (Intergen Company, Oxford, UK) according to the manufacturer’s instructions. To define the sensitivity of the method and the semiquantitative relationship between protein concentration and ladder band intensity, different amounts of protein extract (from 0.1 to 2 µg) were used for each purified lymphocyte culture and for all assays. The appropriate protein concentration to produce a linear response was 1 µg of the total protein. In all cases, reaction products were amplified in the presence of a 36 bp internal TRAP assay standard (ITAS), and each extract was tested for RNase sensitivity, incubating samples with 20 ng RNase (Sigma) for 30 min at 37°C before extension/amplification reaction. Telomeric repeat amplification protocol assay included a sample without cell lysate as negative control and a positive control sample provided by the kit. Telomere length was evaluated by Southern blot as previously reported (Biroccio et al., 2002). Briefly, total DNA was isolated using standard procedure. For each sample, 15 µg of DNA were digested with 40 U of HinfI and electrophoresed on 0.8% agarose gel. DNA was denatured, neutralised, transferred to a nylon membrane (Hybond N, Amersham International, Buckinghamshire, UK) and cross-linked with ultraviolet light. The membrane was hybridised with 5'-end [32P]-deoxyadenosine triphosphate-labelled telomeric oligonucleotide probe (TTAGGG)_n at 42°C for 4 h in a rapid hybridization buffer (QuikHyb Hybridization Solution, Stratagene, La Jolla, USA). After washing, the filters were autoradiographed (Hyperfilm-MP; Amersham) with an intensifying screen at ~80°C for 24 h. The autoradiographs were scanned and the mean telomere length was calculated as reported by Harley et al (1990).

Cell cycle analysis and detection of apoptosis

The percentage of cells in the different phases of cell cycle was determined by evaluating DNA content. Treated and untreated cells (2 x 10^6) were collected at 72 h of culture, washed with PBS and fixed with 2 ml of 70% ethanol. After washing twice in PBS, the cells were stained with a solution containing 50 µg ml^-1 propidium iodide (PI) and 75 kU/ml RNase in PBS for 30 min at room temperature. A total of 20 000 events per sample were acquired by FACS Calibur (Becton-Dickinson) using a doublet discrimination module (DDM). The percentages of cell cycle distribution and subG1 apoptotic cells were calculated on linear PI histograms using the mathematical software ModFit. Apoptotic cells were also detected by using the APO-BRDU™ kit (BioSource International, Camarillo, California), a terminal deoxynucleotide transferase (TdT)-mediated Br-dUTP nick-end labelling (TUNEL), according to the manufacturer’s instruction. Briefly, 2 x 10^6 of treated and untreated cells were collected at 72 h of culture, washed with PBS and fixed with 2 ml of 70% ethanol. The fragmented DNA was revealed incubating the samples with the labelling reaction mix for 1 h at 37°C; the BrdU-labelled cells were then stained with a fluorescein-labelled anti-BrdU antibody solution for 30 min at room temperature, in the dark. To determine DNA content, a PI/RNase solution was added to FITC-labelled cells and incubated for 30 min at room temperature, in the dark.

Statistical analysis

Statistical evaluation of apoptosis and cell cycle data was performed by Student’s t-test.

RESULTS

Telomerase activity and telomere length in AT lymphocytes expressing TCL1

In order to study the correlation between chromosome end-to-end fusions (tas) and telomere metabolism, we evaluated telomerase activity and telomere length in PHA-stimulated lymphocytes characterised by different tas frequency. The cells used were: AT lymphocytes with TCL1 expression (AT94-1), which are characterised by a high frequency of spontaneous tas (Table 1); Ataxia telangiectasia lymphocytes without TCL1 expression (AT95-1 and AT95-2) showing sporadic tas; and normal lymphocytes, with no detectable tas. The telomerase activity was evaluated by a semiquantitative TRAP assay. Figure 1 shows that the loss of the ATM function alone determines a marked decrease in telomerase activity (see AT95-1 and AT95-2), while the expression of TCL1, characteristic of AT94-1 clonal lymphocytes, is able to restore telomerase activity up to the level observed in normal lymphocytes. The internal telomerase assay standard (ITAS) was amplified to the same extent in all cell cultures, excluding the presence of Taq polymerase inhibitors in AT lymphocytes without TCL1 expression.

We then tried to determine whether the increase of telomerase activity observed in AT cells expressing TCL1 was associated with reduced telomere shortening. Terminal restriction fragments (TRFs) were obtained by Southern blotting in the same cells analysed by TRAP assay (Table 2). TRFs in AT lymphocytes without TCL1 expression were shorter (mean value 13.1 kb) than in normal lymphocytes (mean value 15.6 kb). AT94-1 lymphocytes (expressing TCL1) showed a telomere length range superimposable to that observed in AT lymphocytes not expressing TCL1, although these two kinds of AT cells have different telomerase activity and different tas frequency.
Decreased apoptosis and restoration of cell cycle progression in AT lymphocytes expressing TCL1

In order to ascertain whether the differences in telomere metabolism in cells that have lost the ATM function are due to different rates of proliferation and/or apoptosis, we analysed these two features comparing AT lymphocytes with and without TCL1 expression. A flow cytometric TUNEL assay, performed in a biparametric analysis with DNA content (Figure 2A), was carried out in four different samples of normal lymphocytes, in two AT lymphocytes (AT95-1, AT95-2) and in AT94-1 lymphocytes. Each sample was analysed at least three times. Figure 2B shows the mean values of apoptotic cells (dark grey columns) together with the mean percentages of cells in the different phases of cell cycle (G0/G1, white columns; S-G2/M, grey columns). Ataxia telangiectasia lymphocytes without TCL1 expression (see AT) were characterized...
by a significant \((P < 0.01)\) spontaneous activation of the apoptotic programme \((53.3 \pm 0.4)\) if compared with normal lymphocytes \((17.0 \pm 2.1)\) and showed a strong proliferative block in the G0/G1 phase. The expression of TCLI in AT cells determined a decrease of the apoptotic rate \((32.9 \pm 5.3)\) and was able to restore a ‘normal like’ cell cycle by driving the AT cells in the S phase. In fact, the differences in the percentage of cells in G0/G1 and S–G2/M phases between normal and AT94-1 lymphocytes were not significant \((P > 0.05)\).

We next evaluated the same parameters in the same cells after DNA damage induction by VP16 treatment (Table 3). As expected, in normal lymphocytes \((A^- TCLI^-)\) VP16 treatment induced a significant \((P < 0.01)\) increase of the percentage of cells in S + G2/M compartment and in apoptosis, while we observed no significant \((P > 0.05)\) change for both parameters in control AT lymphocytes \((A^- TCLI^-)\). On the contrary, in AT lymphocytes expressing TCLI \((A^+ TCLI^+)\) VP16 treatment determined an increase of apoptosis rate and of percentage of cells in the S–G2/M compartment comparable to those observed in normal lymphocytes.

### DISCUSSION

In this study, we demonstrated that AT94-1 T lymphocytes have lost the general growth disadvantage caused by the lack of the ATM function. The dramatic reduction of cells in the S and G2 phases of the cell cycle, characteristic of nonclonal AT lymphocytes, was not observed in AT94-1 lymphocytes since these cells showed a cell cycle distribution similar to that observed in normal cells. Furthermore, our results suggest that TCLI expression is correlated with a reduced apoptotic rate in the apoptotic-prone ATM cells \((Duchaud et al, 1996)\). The differences in cell proliferation and survival were also evident in cells treated with VP16, a topoisomerase II inhibitor that acts at premitotic phase and induces apoptosis in mitogen-activated T lymphocytes \((Sinkule, 1984; Ferraro et al, 2000)\). Indeed, when damaged with VP16, only ATM TCLI lymphocytes were able to show an increase, both in apoptosis rate and in percentage of cells in the S–G2/M compartment, similar to that observed in VP16-treated normal lymphocytes \((ATM^- TCLI^-)\).

We also showed that AT94-1 cells exhibited telomere shortening, increased telomerase activity and chromosomal instability, especially telomeric end-to-end fusions \((tas)\). The coexistence of telomere instability and telomerase activation in AT94-1 cells can be explained by taking into account that telomerase activity does not necessarily result in telomere elongation, in agreement with previous studies \((Metcalfe et al, 1996)\). Indeed, telomerase might have a dual function for telomeres, protection and elongation, and could therefore promote cell survival and growth, independently of net telomere elongation \((Zhu et al, 1999; Blasco, 2002)\). We also observed that endogenous expression of telomerase in AT94-1 cells is unable to correct the cytogenetic phenotype of telomere instability (i.e. \(tas\)). In agreement with this, Wood et al \((2001)\) showed that the ectopic expression of the human telomerase gene \(hTERT\) in AT fibroblasts does not correct the telomere end-association defect, despite maintaining/extending telomere length. Thus, \(tas\) frequency could be dependent on ATM loss, but is telomerase independent. In fact, as suggested by Pandita and Dhar \((2000)\), ATM could be involved in telomere-mediated function through telomere–nuclear matrix interaction. Therefore, AT94-1 lymphocytes regain telomerase activity despite high frequency of \(tas\), show low spontaneous apoptosis rate and restore cell cycle progression. It is then TCLI expression that confers these advantages to ATM cells. Even though we cannot exclude that other undetected genetic alterations might be present in the clonal cells, TCLI seems to play an important role in these features. This idea is consistent with the observation that TCLI is uniquely expressed in AT94-1 lymphocytes and not in other AT or normal T lymphocytes \((Narducci et al, 1995, 2000)\) and that it has a proliferative and antiapoptotic role as demonstrated by recent biochemical findings and studies in animal models. More specifically, biochemical evidence demonstrates that TCLI acts in vivo as a coactivator of the Akt protein, enhancing its kinase activity and mediating its nuclear translocation \((Pekarsky et al, 2000; Kunstle et al, 2002)\). Akt, in turn, has a central role in the regulation of several signalling pathways controlling cell survival and proliferation in T cells. Indeed, activated Akt enhances human telomerase activity through phosphorylation of the hTERT subunit \((Kang et al, 1999)\), inhibits the proapoptotic factor Bad \((Nicholson and Anderson, 2002)\) and, in T lymphocytes, has a key role in G1–S phase progression through the regulation of pRB \((retinoblastoma protein)\) phosphorylation \((Brennan et al, 1997)\). The same pathway Akt/pRB/E2F is required also to protect activated peripheral T cells from bcl-2-independent apoptosis \((Lauder et al, 2001)\). The overexpression of this gene in T or B lymphocytes of transgenic mice induces, respectively, a T-PLL, B-CLL or mature B-cell lymphoma \((Virgilio et al, 1998; Bichi et al, 2002; Hoyer et al, 2002)\). Interestingly, Fears et al \((2002)\) reported that one cell line, derived from a relapse in a child with acute lymphatic leukaemia \((ALL)\), showed TCLI overexpression and a much higher rate of proliferation when compared to a previously established cell line from the same patient. The increase in TCLI expression correlates with disease progression and more aggressive phenotype in this child.

In conclusion, clonal T cells carrying t(14;14) chromosomal translocation and expressing TCLI seem to be able to restore some of the biological properties that are lost in AT cells; this might explain why these T cells have greater expansion and survival rate and are most often observed in AT patients. This assumes even more relevance if we consider that a similar situation of ATM biallelic loss and TCLI overexpression, other than in AT patients, exists also in several sporadic human cancer types, such as T-PLL, B-CLL and mantle cell lymphoma, suggesting that these two genes cooperate in the development of certain lymphoid tumours.

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