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QUARTERLY

Proliferation and apoptosis of human T cells during replicative senescence — a critical approach★

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Normal human T lymphocytes growing in culture undergo replicative senescence. Previously, we have shown that in our conditions polyclonal T cells cease proliferation after about three weeks (Radziszewska *et al.*, 1999, *Cell Biol. Int.* 23, 97–103). Now we present results of a more detailed analysis of *in vitro* growth *as well as phenotypic changes of T cells. Cell cycle analysis showed that about 20% of cells were in the S phase untill the 17th day of culture (young cells). The highest number of mitotic cells (phase G_2*/M; 10%) was observed during the first week of culture. All not dividing senescent cells were stopped in the G_1 phase (after the 30th day of culture). The sub-G_1 fraction which represents apoptotic cells did not exceed 8% during the whole period until the 30th day of culture. During *in vitro* T-cell growth, a rather rapid selection to CD3+CD8+ cells occurs. In the presenescent (between the 17th and 30th day) and senescent populations the majority of cells (above 90%) were CD8 positive. We also have checked the expression of α-chain interleukin-2 (IL-2) receptor (CD25). In young and presenescent cells about one third of cells was CD25 positive, but only 15% in the pool of senescent cells. Immunoblotting analysis of p16 protein recognized previously as a marker of senescent T cells, showed its highest and transient expression in presenescent cells. A critical review of the polyclonal T cell replicative senescence model is presented.

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Abbreviations: PHA, phytohemaglutinin; CD3, T cells; CD19, B cells; CD8, cytotoxic T cells; CD16/CD56, natural killers cells; CD25, a chain of IL-2 receptor; PBL's, peripheral blood lymphocytes; TCR, T cell receptor; CD95(Fas), receptor which provide signal to apoptosis.

Deterioration of the immune system with aging is believed to contribute to increased incidence of infection, autoimmune diseases and cancer. Deregulation of the immune system may appear at many different levels including: stem cell defect, thymus involution, changes in antigen presenting cells, signal transduction, changes in cell surface receptors etc. A dramatic decline in immune function involves both B and T cells [1]. The crucial events in the function of T cells are both proliferation and apoptosis. For many years research interest has been focused on T cell proliferation, resulting in a number of reports which prove poorer response of the cells to different mitogens during aging. Although recently also apoptosis has been recognized as an important factor in shaping and maintaining the T cells repertoire, the propensity of the senscent cells to undergo cell death is far from being elucidated [2, 3].

Research concerning aging of immune system is carried out in two complementary fields: *ex vivo* and *in vitro* studies. Studies on *in vitro* aging, based on serial cultivation of cells (mainly fibroblasts, epithelial cells and recently lymphocytes) in defined artificial conditions have provided a large contribution to elucidation of the molecular mechanism leading to the cessation of cell division. It is also believed that, to a certain extent *in vitro* senescing reflects the processes *in vivo* [4].

The results reported by a number of groups demonstrated that normal human T lymphocytes have a finite lifespan even if the number of doublings varies depending on culture conditions [5]. Certain genetic and phenotypic changes observed during replicative senescence of T lymphocytes prove that these cells really do exhaust their proliferation limit and resemble the T cells in aged individuals. In senescent T cells, the shortening of telomeres [6], accumulation of cycline-dependent kinases (Cdks) inhibitors, namely p15 and p16 [7] and progressive loss of CD28, a costimulatory molecule that is required for optimal activation and proliferation following engagement of T cell receptor [8], has been documented in T-cell cultures. Short telomeres as well as a relative increase of CD28 negative cells are also observed in lymphocytes derived from very old people (centenarians) [9, 10]. Moreover, CD28 negative cells have been shown to be less prone to apoptosis induced by different stimuli in comparison with young and CD28 positive cells [11]. Also our previous results indicated that non proliferating senescent T cells were less prone to UVC induced apoptosis than proliferating young cells [12]. Data concerning apoptosis of T cells derived from old subjects are not so clear and depend very much on the T-cell subset studied [13].

Previously we have shown that in our conditions human polyclonal interleukin-2-dependent T cells cease to proliferate after about three weeks [12]. Now, we present results of a more detailed analysis of *in vitro* growth, phenotypic changes and capacity to undergo spontaneous apoptosis of T cells.

MATERIALS AND METHODS

Cell culture. Human peripheral blood lymphocytes (PBL's) from healthy 20–30 year old volunteers were isolated by standard Ficoll-Hypaque gradient centrifugation. Cells were taken up in RPMI1640 medium supplemented with 10% foetal calf serum (Gibco, U.K.) and cultured at 37°C in 5% CO_2 humidified incubator. For the generation of T-lymphocytes lines, PBL's were activated for 3 days with phytohemaglutinin (PHA, 10 μg/ml) (Sigma, Germany) and then cultivated in a medium enriched with human recombined interleukin-2 (IL-2, 10 U/ml, R&D, U.K.) at a density which provided conditions for logarithmic growth.

Cell cycle analysis. Distribution of lymphocytes in cell cycle phases during *in vitro* growth was analysed by cell staining with 4′,6′-diamidino-2-phenylindole (DAPI) and

sulforhodamine. The percentage of cells representing G_1, S and G_2/M phases was established using MacCycle Programme and the mean numbers of cells in a given phase were plotted against the culture duration.

Immunophenotyping of T lymphocytes. Immunophenotyping of T cells was performed by Two Colour FACS analysis according to Becton Dickinson Procedure and using Simultest antibodies conjugated with fluorescein isothiocyanite (FITC) or phycoerythin (PE).

Expression of CD25 in the CD8-positive subpopulation of T lymphocytes was analysed using anti-CD25 conjugated with FITC and anti-CD8 conjugated with peridinin chlorophyll protein (PerCP).

Measurement of p16 expression. Expression of p16 protein was measured by immunocytochemical staining of cells. Cells were centrifuged on a cytospin and then fixed in 4% paraformaldehyde for 1 h for immunocytochemical staining. The cells were washed several times in phosphate buffered saline (PBS), and treated with 0.3% hydrogen peroxide for 15 min. Unspecific binding was blocked by cell incubation with 5% solution of normal goat serum in PBS for 30 min. Then, the lymphocytes were incubated for 1 h with anti-p16 primary specific polyclonal antibody diluted 1:1000 (Santa Cruz Biotechnology). Then the cells were treated for 1 h with avidin-biotin complex and the colour reaction was developed using diaminobenzidine (DAB) chromogen (Sigma). Every step was preceded by extensive washing in 0.3% solution of Triton X-100 in PBS, at room temperature.

Apoptosis measurement. Apoptosis measurement was done by flow cytometry and Hoechst 33258 staining. Flow cytometry analysis was done after cell staining with DAPI (1 μg/ml) and sulforhodamine (20 μg/ml) (from Molecular Probes) at 4°C. For each time point 1×10^6 cells were stained. Cells were analysed for DNA (DAPI) and protein (sulforhodamine) content on FACS Vantage (Becton-Dickinson) using Cell-Quest software (Becton-Dickinson).

The results were confirmed by microscopic observation after Hoechst 33342 dye staining (5 μg/ml, Molecular Probes).

RESULTS

Growth characteristics of T cells

Cell cycle analysis (Fig. 1) showed that about 20% of cells were in S phase untill the 17th day of culture. The number of mitotic cells (phase G_2/M; 10%) was the highest during the first week of the culture. All not dividing senescent cells were stopped in G_1 phase. These results indicate that cessation of T cell growth occurs gradualy and that growth retardation is preceded by an S phase of longer duration. According to these data as well as the data showing thymidine incorporation to DNA and population doubling number per day (not shown), the cells from 0 untill the 17th day were described as young, between the 17th and 30th day as presenescent, and afterwards when all the cells were not dividing, they were described as senescent.

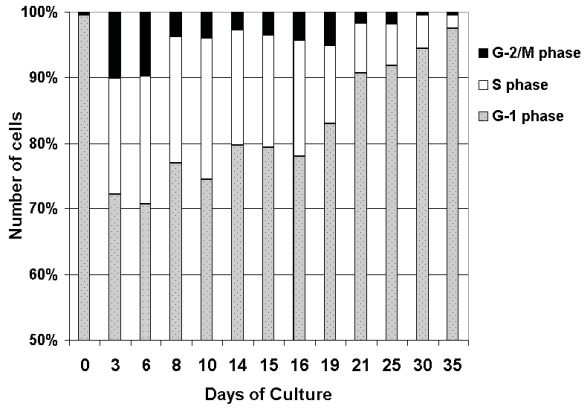

Figure 1. Cell cycle analysis of T cells.

Cell distribution in the cell cycle was analysed by DAPI and sulforhodamine staining. The percentage of cells representing G_1, S and G_2/M phases was established using MacCycle Programme and the mean numbers of cells in a given phase were plotted against the culture duration.

Spontaneous apoptosis of T cells

Once all cells stopped dividing, one could expect that a massive apoptosis would occur at the end of culture. Nonetheless, we observed that the sub-G_1 fraction did not exceed 8% during the whole period until the 30th day of culture (Fig. 2). The only exception was the 3rd day of culture on which the cells were PHA-treated (15% of sub-G_1). Also morphological observation after Hoechst staining showed that senescent cells were not apoptotic (Fig. 2).

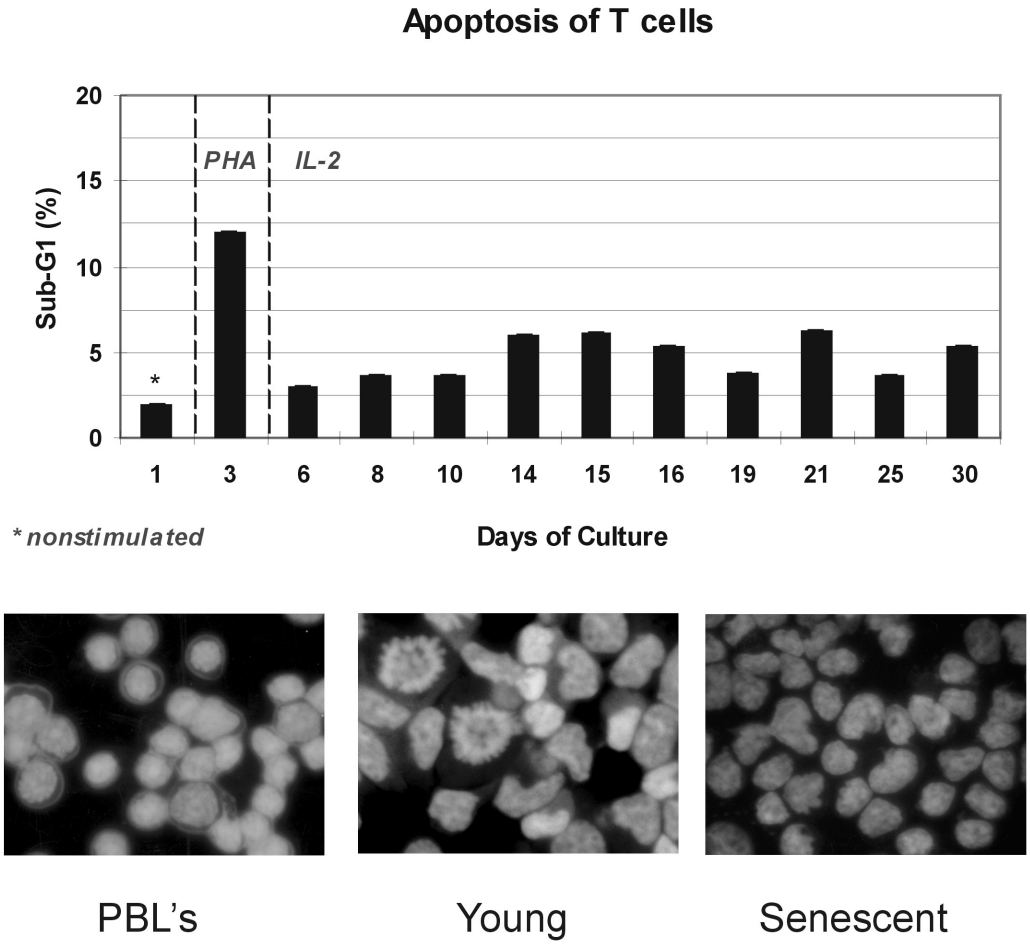

Figure 2. Apoptosis of T cells.

The upper panel shows the percentage of sub-G_1 fraction in T-cell population. The lower panel show cells, as indicated, stained with Hoechst dye.

Phenotype changes of T cells

In young, presenescent and senescent Ficoll-isolated PBL's from blood, the presence of surface phenotypes such as CD3 (T cells), CD19 (B cells), CD4 (T helper cells) CD8 (T cytotoxic cells), CD16/CD56 (natural killers) and CD25 (T cells expressing α chain of IL-2 receptor) were estimated by flow cytometry (Table 1). As early as after PHA stimulation (young) practically all cells were CD3 positive. During *in vitro* growth of T lymphocytes, a quite rapid selection to CD3+CD8+ cells occurred. In the pool of young cells we observed 30% of CD4+, but in the presenescent and senescent populations the majority of cells (above 90%) were CD8+. Senescent T cells, despite having IL-2 in the medium, did not divide any longer. We checked also the CD25 expression, which is a good marker of early activation. In young and presenescent cells

about one third of the cell population was CD25 positive, but only 15% of CD25 was present in the pool of senescent cells. The same distribution of CD25 positive cells as in whole population, was observed in the CD8+ subpopulation (Fig. 3). Although we did not observe significant differences in the percentage of CD25+ cells between the yound and presenescent subpopulations (Table 1), the analysis of cell brightness reflecting the amount of anti-CD25 antibodies bound to the surface of a given cell revealed that the number of CD25 molecules on a single young cell was higher than that observed on presenescent and senescent cells.

Table 1. Phenotype changes in lymphocyte population during replicative senescence

	CD3	CD19	CD4	CD8	CD16CD56	CD25
PBL's	59	16	27	31	15	nd
Young	99	<1	30	60	4	31
Presenescent	99	<1	7	93	2	35
Senescent	99	<1	4	92	<1	15

Immunophenotyping of T cells was performed by Two Colour FACS analysis according to Becton Dickinson Procedure and using Simultest antibodies conjugated with fluorescein isothiocyanite (FITC) and phycoerythin (PE).

p16 Expression in T cells

Only a few biomarkers of T cells senescence have been defined. One of them is a reduced expression of CD28+, induction of acid β-galactosidase and accumulation of p16 protein [5, 7]. We measured the expression of p16 in PBL's, and in young, presenescent and senescent cells (Fig. 4). Surprisingly we found that an induction of p16 in presenescent cells was not transient and decreased in senescent cells to the level detected in PBL's.

DISCUSSION

Replicative senescence of normal cells is a very useful model of aging which provides information about molecular changes responsible for cessation of proliferation and which can be referred to the phenomena occurring *in vivo* [14]. Molecular analyses have identified many changes in gene expression and activity of their products in the cells undergoing replicative senescence. Senescent cells become blocked at the G_1 phase of the cell cycle and are unable to enter the S phase. Control of the cell cycle progression is based on cycline dependent kinases (Cdks) which are positively regulated by cyclines and negatively by protein inhibitors belonging to the INK4 (p15, p16, p18, p19) and Cip/Kip (p21, p27, p57) families. Moreover, tumour suppressor proteins pRb and p53 are also involved in this control system. It should be emphasised that most of this information was obtained from experiments performed on fibroblasts [15]. Later some of these observations were also confirmed on human T cells undergoing replicative senescence [16, 17]. Recently, it has been shown that both p15 and p16 proteins accumulate with aging of PHA-stimulated T cells in culture, and that there is increased binding of p16 to its target Cdk 6 kinase [7]. Surprisingly, our results did not confirm this observation, as in our hands, transient elevation of p16 was observed during culture (in presenescent cells). This could suggest that among the presenescent CD8+ cells there are some which really are senescent and may successively die, but the rest of them although they are not dividing can restore capacity to proliferate. Especially, that still 15% of not dividing senescent cells are CD25 positive. It is also possible that lymphocytes had ceased proliferation because they

were not restimulated *via* the T cell receptor (TCR). Our preliminary reassert has shown that it is possible to activate a small population of T lymphocytes after 30th days of culture. For this reason it would be more proper to focus on the research with the use of monoclonal cultures of T lymphocytes undergoing replicative senescence. Indeed, the group of Pawelec demonstrated that certain clones differ in their proliferation limits, but anyway all of them finally stop dividing in culture [18]. The second solution is an as broad as possible phenotype analysis of polyclonal cultures. One candidate is, for example, the CD28 phenotype as it was shown that cessation of proliferation was tightly connected

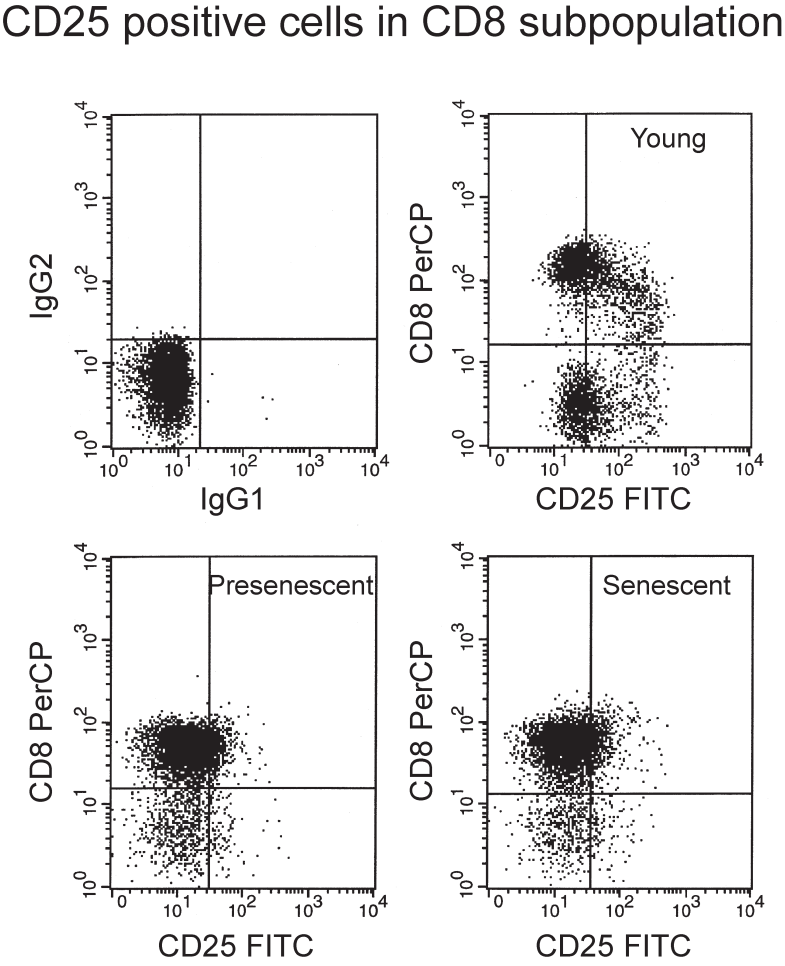

Figure 3. Distribution of CD25 positive cells in the population of CD8 positive lymphocytes.

Expression of CD25 in the CD8 positive subpopulation of T lymphocytes was analysed by the immunofluorescence assay performed by incubating the T cells with anti-CD25 (FITC, FL-1) and anti-CD8 (PerCP, FL-3) according to Becton Dickinson procedures for Simultest™. Double negative control of cells stained with IgG1 and IgG2 is also shown. For other details see Methods.

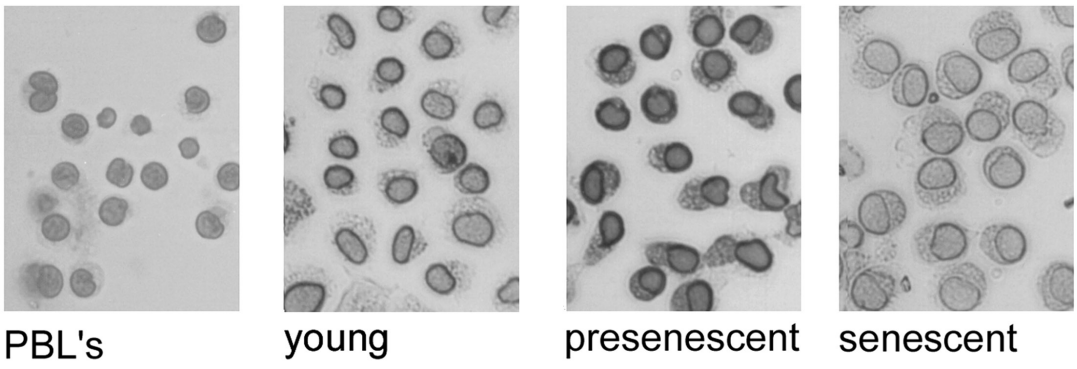

Figure 4. Immunostaining of p16 protein in the cells at different stages of culture as indicated.

with loss of the CD28 coreceptor molecule on CD8 positive cells [19]. Hathcock *et al.* [16] have shown, that optimal induction of telomerase activity and proliferative response by T cells appear to require both TCR-signals and costimulatory signals that can be provided by CD28-B-7 interaction. Although in our conditions a rather rapid selection to CD8 positive cells occurred, the information about CD28 expression is needed and this is at present under evaluation.

The propensity of senescent cells to undergo apoptosis still remains an open question. Impaired T-cell apoptosis *in vivo* has been reported during both normal aging and in autoimmune diseases. On the other hand, Spaulding *et al.* [11] showed that replicative senescence in CD8 positive T cell cultures was associated with significant resistance to apoptosis induced by various factors. This discrepancy can be explained as follows: In a polyclonal culture, selection of CD+8CD28-CD95 cells which are resistant to apoptosis is usually observed. *In vivo,* even if some relative increase of this subset does occur, the number of CD95 positive, namely Fas expressing cells is growing up thus overlapping resistance to apoptosis [20]. The relatively stable and small number of cells undergoing apoptosis in our conditions showed that, during culture the cells did not acquired the propensity to undergo spontaneous apoptosis. However, our experiments did not provide information about CD95 expression and cell capacity to undergo induced cell death. Nonetheless, according to our previous data as well as the results obtained by others we can conclude that a certain phenotype of cells senescing *in vitro,* namely CD8+CD28-, is resistant to apoptosis [11, 12].

To sum up, human IL-2- dependent T-cell cultures can provide important information about cell proliferation and apoptosis and can be used as a model of cell senescence, although due to physiological differences observed among different cell subsets, careful analysis of the cell phenotype is necessary.

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