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H1 Histone Subtype Constitution and Phosphorylation State of the Ageing Cell System of Human Peripheral Blood Lymphocytes

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Abstract

Until a few years ago, the H1 histones were exclusively considered to be the architectural proteins of chromatin involved in chromatin condensation. However there is now increasing data to support the hypothesis that the H1 subtypes are involved in genomic integrity and that they may have unexpected functional roles in various biological processes such as in differentiation and DNA repair, apoptosis and lifespan. Moreover, the H1 histones are phosphorylated to a great extent. Recent work has implicated phosphorylation of H1 in the regulation of chromatin remodeling. In light of the fact that chromatin reorganization and heterochromatin formation has been shown to take place during ageing and senescence, in the present investigation, we have analyzed the changes that take place in the somatic H1 linker histone subtype profile and their phosphorylation states in human peripheral blood lymphocytes as a function of donor age. Results from this work show that there is a significant age-related dephosphorylation of H1.4 and H1.5 and an increase in the heterochromatin protein HP1 α as a function of donor age. These results indicate that dephosphorylation of H1 histones may be related to an increase in senescence-associated heterochromatin formation during the in vivo ageing of human peripheral blood lymphocytes.

1. Introduction

The H1 linker histone family has been extensively studied for more than three decades. Earlier investigations analyzed the histone H1 subtype composition in many cell and tissue systems, including lymphocytes, fibroblasts, spermatocytes, non dividing and/or quiescent cell/tissue systems, dividing somatic cell types (reviewed in Lennox and Cohen, 1988), differentiating cell systems (Pina and Suau, 1987), leukemic cell types (Mannironi et al., 1987), etc. This large body of work revealed the extensive diversity of the H1 subtypes that are present in varying proportions depending on the species and/or the cell/tissue system. In mammals, there are five H1 somatic subtypes, H1.1, H1.2, H1.3, H1.4 and H1.5 (Eick, et al., 1989; Albig et al., 1991; 1993; 1996b; Drabent et al., 1995a, reviewed in Doenecke et al., 1997), the replacement H1 subtype, H1^o (Gjerset et al., 1981; 1982), the ubiquitous H1x (Yamamoto and Horikoshi, 1996; Happel et al., 2005), the testis-specific subtypes, H1t, H1T2 and H1LS1 (Meistrich et al., 1985; 1994; Drabent et al., 1996; Martianov et al., 2005; Yan et al., 2003) and an oocyte-specific subtype (Tanaka et al., 2001). Moreover, classic in vitro investigations analyzed the role of this histone family in chromatin structure and function. Results from this extensive line of work suggested that the H1 histones were exclusively architectural proteins of chromatin involved in chromatin condensation (e.g., Allan et al., 1980; Pruss, 1996; Crane-Robinson, 1997; Bednar et al., 1998; Carruthers and Hansen, 2000). These in vitro properties of the H1 histones led to the speculation that these proteins function as global repressors of transcription. However, their evolutionary conserved heterogeneity (Parseghian and Hamkaló, 2001) and results from more recent in vivo and in vitro studies have come to challenge and reappraise this view (Woodcock et al., 2006). H1 subtype depletion studies from Skoultchi's group (e.g., Fan et al., 2005) and others have raised additional questions as to the functional role of the H1 subtypes. There is now increasing data to support the hypothesis that the H1 subtypes are involved in genomic integrity and that they may have unexpected roles in various biological processes (Harvey and Downs, 2004) and may act not only as negative regulators, but also as positive regulators of

a certain subset of genes (Brown, 2003). Recent studies have also assessed the binding affinities of all the subtypes and their dynamic mobility within chromatin as well as their localization and association with specific euchromatic and heterochromatic regions or domains (Th'ng et al., 2005).

Moreover the H1 subtypes undergo phosphorylation which culminates at cell division and the original correlation has been that this cell cycle-related increase in H1 phosphorylation is related solely to mitotic chromosome condensation. It has been postulated that H1 phosphorylation weakens the binding of H1 to chromatin, allowing the profound structural changes that accompany chromosome condensation (Bradbury et al., 1973; 1974). However in some biological systems, H1 phosphorylation is uncoupled from mitosis and chromosome condensation. In these systems condensed chromatin is enriched in dephosphorylated forms of H1 (Roth and Allis, 1992). For example in *Tetrahymena*, changes in H1 phosphorylation appear to occur whenever there are marked changes in gene expression due to altered physiological conditions, supporting a role for H1 phosphorylation in transcriptional regulation (Roth, et al., 1988; Lin, et al., 1991). Indeed in *Tetrahymena*, data have confirmed the role of histone H1 phosphorylation in the transcriptional regulation of certain genes (Dou and Gorovsky, 2000). Furthermore, using antibodies selective for phosphorylated H1 histones, enrichment of phosphorylated H1 histones in transcriptionally active chromatin has been observed (Lu et al., 1995; Chadee et al., 1995). Recent in vitro studies have also implicated phosphorylation of H1 in the regulation of chromatin remodeling (Horn et al., 2002; Vicent et al., 2003). Phosphorylation seems to stimulate the dynamic exchange of linker histones between chromatin fibers by increasing the rate of dissociation of H1 from chromatin (Dou et al., 2002; Contreras et al., 2003). This may provide a mechanism by which phosphorylation can affect H1 function in vivo. In other words, the more recent results have shown that dephosphorylation of H1 is associated with the maintenance of H1 subtypes on chromatin and the formation or stabilization of condensed structures, while H1 phosphorylation is associated with transient removal or dynamic exchange of H1 subtypes and the destabilization of condensed structures. How, then, is this reconciled to S, G2 and M phase accumulation of H1 phosphorylation and chromatin condensation for mitotic

chromosome formation? One proposal is that H1 hyperphosphorylation itself might serve as a mitotic “trigger” and not the actual physical means for condensation (Roth and Allis, 1992). While this may or may not hold true, the recent evidence cited above supports the hypothesis and model originally proposed by Roth and Allis (1992) that rather than playing a direct physical role in the chromosome condensation process, H1 phosphorylation actually leads to a transient decondensation which allows specific chromosome condensation factors (for example, topoisomerase II) to gain access to specific DNA sequences. This would also account for the more global role of H1 phosphorylation and dephosphorylation in chromatin decondensation / condensation, which would allow or restrict, respectively, access to transcriptional DNA binding factors. Thus, along with other histone modifications (such as acetylation and deacetylation), as well as many other chromatin structural factors, transcriptional regulation or the reorganization of euchromatin and heterochromatin regions (chromatin remodeling), may take place.

These data point towards the fact that specific H1 subtypes and their phosphorylation states may have specific functional roles in chromatin remodeling events which are responsible for transcriptional regulation that takes place during various biological processes such as development, differentiation, DNA repair (Downs et al., 2003), apoptosis (Konishi et al., 2003; Okamura et al., 2007) and lifespan (Barra et al., 2000).

In light of the evidence that specific H1 subtypes associate with specific chromatin regions and their possible functional involvement in chromatin reorganization and remodeling events that regulate transcriptional activity exclusively or in conjunction with their phosphorylation states, we were interested in analyzing changes that may take place in the somatic H1 histone subtype constitution during the *in vivo* ageing of human peripheral blood lymphocytes. The incentive for this line of research is the significant role that chromatin reorganization events are known to have during the ageing process and senescence. The role of heterochromatin has been one of the main objects of research and debate regarding its genetic contribution to the ageing phenomenon. However although changes in the state of chromatin have been described by many investigators in various ageing cell systems, the

information gained so far has been controversial. Models on how chromatin reorganization occurs during ageing have been proposed in which there is a rearrangement of euchromatin (transcriptionally active or transcriptionally competent) and heterochromatin (condensed, transcriptionally silent) regions (Imai and Kitano, 1998). Since ageing is a programmed event, this means that certain genes must shut down (e.g., cell cycle-related) whereas others must be activated (senescence-associated). Research results in this area have related ageing to both increases and decreases in heterochromatin. Howard (1996) as well as Villeponteau (1997) have described results which indicate that there is a progressive recession of heterochromatin-like domains. Howard postulates that this may be associated with memory-dependent maintenance of repressive heterochromatin domains. Errors in maintenance, resulting from the failure of repressive chromatin domains to reassemble fully following DNA replication, accumulate during the proliferative lifespan, ultimately triggering a cell cycle checkpoint and consequent irreversible cell cycle exit. Thus irreversible replicative arrest (senescence) may be related in a fundamental way to the formation and subsequent cell cycle-coupled maintenance of heterochromatin domains. Narita et al. (2003) have shown in senescent fibroblasts that there is an accumulation of distinct chromatin structures (senescence-associated heterochromatin foci, SAHF) which are enriched with heterochromatin proteins (HP1) and contribute to senescence-associated changes in gene expression (e.g., cell cycle checkpoint proteins and cell cycle arrest) and the permanence of the senescent state. Lezhava (2001) found that during *in vivo* ageing of human lymphocytes progressive heterochromatinization occurs, which he describes as a type of facultative heterochromatin reformation. In fact lymphocytes from individuals of 70 years and over exhibit intensive heterochromatinization of their chromatin.

At the beginning of the 1960s, Hayflick and Moorhead (1961) discovered that normal diploid fibroblasts have a limited replicative lifespan and until recently replicative senescence was studied exclusively in *in vitro* fibroblast cell culture systems. However, human peripheral blood lymphocytes are among the best characterized *in vivo* ageing cell systems and as such are an ideal biological material with which to study *in vivo* ageing. Since chromatin reorganization and heterochromatin formation has been shown to take place

in ageing lymphocytes, the present investigation has analyzed the changes that take place in the somatic H1 linker histone subtype profile and their phosphorylation states in lymphocytes as a function of donor age. This line of investigation is unique since though much work has been accomplished on the potential role of the differentiation-associated histone H1 replacement subtype, H1^o, in differentiating (reviewed in Zlatanova and Doenecke, 1994) and ageing (Tsapali et al., 2000a; 2000b; 2001; Sourlingas, et al., 2002; Kypreou et al., 2004) cell systems, much less work has been accomplished as to the potential role of the somatic subtypes and their phosphorylation states in in vivo biological processes such as those mentioned above, and no work, to date, as to a potential involvement of the somatic subtypes and their major post-translational modification, phosphorylation, in ageing cell systems. The results obtained in this investigation are compared to existing information from in vivo and in vitro studies regarding the association and relationship of the somatic H1 subtypes and their states of phosphorylation to eu- and heterochromatin domains and/or decondensed and condensed chromatin.

2. Materials and methods

2.1. Blood donors

A total of 26 healthy donors from four different age groups were used in this study: young, 23-30 years old (7 donors), mid-aged, 38-50 years old (5 donors), senior, 60-65 years old (5 donors) and elderly, 73-95 years old (9 donors). The young, mid-aged and senior donors were PhD students and staff members of the Biology Institute of the National Centre for Scientific Research, "DEMOKRITOS" and the elderly donors were relatives of the students and staff members. Health status of the donors was ascertained by interview and assessment of recent laboratory tests. Donors having passed a recent inflammation or with a chronic disease and/or on some type of drug therapy were excluded from the study. Informed consent was obtained from all donors and the study conformed to the rules of the Declaration of Helsinki.

2.2. Blood collection and cell culturing

Forty milliliters of venous blood were drawn between the hours of 8:00-10:00 am in tubes containing 1 mg of ethylenediaminetetraacetic acid (EDTA)/ml blood. Blood was diluted with HANKS' balanced salt solution (1:1), overlaid onto a Ficoll-Hypaque gradient (1:1) and centrifuged (2000 rpm, 30 min). The interphase band was collected, washed with HANKS' and centrifuged (1800 rpm, 10 min). Cells were counted on a Neubauer hemocytometer after resuspension of cell pellet in RPMI 1640 complete medium containing 10% fetal bovine serum. Density of cell suspensions was 10^6 cells/ml. Cell viabilities were ascertained for all samples using the trypan blue exclusion method. In all cases, cells from the interphase band were 100% viable. Mitogenic stimulation was accomplished by adding to each culture 5 µg phytohemagglutinin (PHA)/ ml culture. Cells were harvested 72 hours after stimulation.

2.3. Histone extraction from Peripheral Blood Lymphocytes (PBL)

For the purification of H1 histones by extraction with perchloric acid, the lymphocyte pellets were resuspended in phosphate-buffered saline containing the protease inhibitor cocktail “Complete” (Roche Diagnostics, Mannheim, Germany). Perchloric acid (PCA) was added to a final concentration of 0.83 M, and cells were lysed and extracted by incubation for one hour on ice. Samples were centrifuged (15 min, 4 °C, 14,000 x g) and the acid-soluble proteins in the supernatant were precipitated with a final concentration of 20 % TCA (w/v) for one hour. After centrifugation (15 min, 4 °C, 14,000 x g), the pellet was washed with cold (-20 °C) acetone and air-dried. The histones were dissolved in 30 mM HCl. The purification of the whole histone fraction was carried out in the same manner as that described above with the exception that the cell pellet was extracted with 0.2 M sulfuric acid instead of 0.83 M perchloric acid.

2.4. Purification of H1 Histones from lymphocytes by gel filtration

H1 histones extracted with 0.83 M PCA, dissolved in 30 mM HCl, were applied onto a Biogel P60 column (Bio-Rad, München, Germany). The column length was 150 mm, the width was 8 mm, the application volume was 170 µl, and the total amount of protein was ~50 µg. The column was eluted with 30 mM HCl. Two hundred µl (200 µl) fractions were collected and 30 µl aliquots of these fractions were separated on 15% SDS-polyacrylamide gels (see 2.7). Other aliquots were used for analysis by capillary zone electrophoresis (see 2.8).

2.5. Purification of H1 histones from the human tumor cell line HL60

The human leukemic cell line HL60 was cultured as previously described (Kratzmeier et al., 2000). Extraction of H1 histones from the HL60 cells was accomplished using perchloric acid in the same manner as that described for the lymphocytes. Purification by gel filtration and reversed-

phase HPLC separation of the different subtypes was carried out as described by Kratzmeier et al. (2000).

2.6 Purification of recombinantly expressed H1 proteins from yeast

Yeast cells were transformed with human H1 subtype genes and induced to express the recombinant proteins, as described by Albig et al. (1998). Extraction of the H1 histones was done using 0.83 M PCA as described by Kratzmeier et al. (1999). The extracted H1 histones were dissolved in 30 mM HCl and further purified by gel filtration using a Biogel P60 column (Bio-Rad, Munich, Germany).

2.7. Immunoblotting

Proteins were separated on 15% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Uniform blotting was checked by staining the nitrocellulose membrane with Ponceau S (Sigma, Taufkirchen, Germany). Blots were probed with histone H3 antibody (#9715, CST, New England Biolabs, Frankfurt, Germany), polyclonal H1x-specific antiserum in a 1:1000 dilution (Happel et al., 2005), a H1.3 antibody (#ab24174, Abcam, Cambridge, UK), a H1.5 antibody (#ab24175, Abcam, Cambridge, UK), a H1.1 antibody (#ab17584, Abcam, Cambridge, UK) or a polyclonal antiserum specific for H1.2 and H1.5 (Ab4112, Stoldt et al., 2007). Furthermore, a polyclonal antibody specific for cyclin A (#CC17, Calbiochem, Schwalbach, Germany) in a 1:40 dilution, an anti-phospho-Histone H2A.X antibody (Ser139) in a 1:10.000 dilution (#05-636, Upstate/ Biomol, Hamburg, Germany) and a HMG17 antibody (kindly provided by M. Bustin, National Cancer Institute, Bethesda, United States) in a 1:3000 dilution were used. The immunoreactive proteins were visualized using the chemiluminescence ECL plus detection system (Amersham Bioscience, Freiburg, Germany) after incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody (#A0545, Sigma, Taufkirchen, Germany) or horseradish peroxidase-conjugated goat anti-mouse antibody (#A9917, Sigma, Taufkirchen, Germany). Stripping of the blotted membranes for reprobing was carried out

by incubating the membranes in stripping buffer (100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 50 °C with occasional agitation. Densitometric quantitation was accomplished using the the ImageJ program (version 1.38b, public domain).

2.8. Capillary zone electrophoresis (CZE) of H1 histones

CZE was performed on a P/ACE MDQ system (Beckman Coulter, Fullerton, USA). The separation conditions are described in Kratzmeier et al. (2000). The capillary type was fused silica with a length of 110 cm and an inner diameter of 50 µm. Separations were performed at the following conditions: temperature, 30 °C; voltage, 18,3 kV; separation time, 60 min; sample injection by pressure; injection time, 10 s; sample protein concentration, ~ 0.5 mg/ml; absorbance detection at 200 nm. Separation buffer was 30 mM H₃PO₄, 60 mM HClO₄, 0.02 % hydroxypropylmethylcellulose, pH 2.0, adjusted with triethylamine. Identification of the peaks was accomplished by mixing experiments using recombinantly-expressed H1 subtypes produced in our lab (see 2.6). Quantification was accomplished using the 32-Karat software version 4.0 (Beckman Coulter, Fullerton, USA).

2.9. Flow cytometry

To estimate the cell cycle distribution, the lymphocytes were prepared according to the instructions of the CycleTest Plus DNA Reagent Kit (#340242, BD Bioscience, San Jose, USA) that was used for fixation and propidium iodide staining. Acquisition of data from the samples was accomplished using a FACSCalibur Flow Cytometer (BD Bioscience, San Jose, USA). Cell cycle distribution was analysed using the software Modfit LT (Verity Software House, Topsham, USA).

2.10. Statistical analysis

The results of the relative area of the CZE peaks for each age group are expressed as the mean and the standard deviation of the mean ($\pm$ S.D.). The one-tailed Student's t-test was used for statistical analysis. A p -value of ≤ 0.05 was considered statistically different.

3. Results

In this investigation peripheral blood lymphocytes (PBL) were obtained from 26 donors of four different age groups: young, 23-30 years old (7 donors), mid-aged, 38-50 years old (5 donors), senior, 60-65 years old (5 donors) and elderly, 73-95 years old (9 donors). The total histone fraction or the H1 fraction was isolated from lymphocytes harvested 72 hours after mitogenic stimulation with PHA. We chose to analyze the cells during this time period because from previous kinetic studies accomplished in our lab using radioactive thymidine, it was ascertained that lymphocytes activated with $5 \mu\text{g}$ PHA/ 10^6 cells/ml begin to enter the S phase approximately 48 hours after activation. At 72 hours, a portion of the cell population is actively synthesizing histones (data not shown). For this investigation we randomly analyzed ^3H -thymidine incorporation from lymphocytes of donors for all age groups and found the expected increase in ^3H -thymidine incorporation in all age groups (data not shown).

3.1. Analysis of H1 subtype composition of PBL by Western blotting

The constitution and a possible age-dependent change of the various H1 subtypes was initially analysed by Western blotting using H1 subtype specific antibodies. The specificity and non cross-reactivity of the H1.1, H1.3 and H1.5 antibodies used in this study (see text below and/or Figure 1B/C) is shown in the blots of Figure 1A using recombinantly expressed H1 subtypes (see Material and Methods, 2.6). The specificity and non cross-reactivity of

H1x and H1.2/H1.5 antibodies also used in this study (see text below and/or Fig. 1B), have been shown previously, again using recombinantly expressed H1 histones for these subtypes, in two recent papers from our group (see Happel et al, 2005 and Stoldt et al., 2007, respectively). Sulfuric acid-extracted H1 histones from donors of four age- groups were separated by SDS-PAGE and transferred to nitrocellulose membranes (Fig. 1B). Figure 1B shows the blots from a representative experiment. Coomassie staining and the blot of histone H3 were used as reference loading controls. As can be seen, the H1.5 subtype does not appear to change with increasing donor age. The levels of H1x also did not change with age. A slight decrease of H1.3 with increasing age was observed. We could not detect any H1.1 in the lymphocytes from donors of any age group. Since H1.1 has been reported to be expressed in lymphocytes (Lennox and Cohen, 1988), our result may be due to the fact that the amount of H1.1 was under the limit of detection with the H1.1 antibody that was used. With respect to the H1.2 subtype, a slight decrease from the young to the mid-aged, as well as a slight increase from the senior to the elderly age-groups, could be detected. However, densitometric quantitation (histogram of Fig. 1D) of the relative intensities (expressed as relative arbitrary units) of the ratios of H1.2 (blot) and the H1.3 (blot) to the total core histone fraction (Coomassie-stained gel) of Fig. 1B showed that there was a clearly observable decrease of H1.3 as a function of increasing age, whereas the H1.2 levels showed minor fluctuations that can not be precisely defined as having an age-related pattern.

To further confirm the decrease of H1.3, the total H1 fraction was extracted with perchloric acid (PCA) and subjected to Western blot analysis. Figure 1C shows the blots from a representative experiment. The amounts of H1 histones that were loaded are shown by Coomassie staining (Fig. 1C). As an additional reference loading control, an immunoblot analysis for H1.5 was first carried out (Fig. 1C), since this subtype was not found to change in the sulfuric acid-extracted histone samples of each age-group shown in the blots of Fig. 1B. The membrane was then stripped and reprobed for detection of H1.3. The amount of H1.3 is clearly decreased in the H1 histone fraction extracted from the elderly PBL (Fig. 1C). In order to further verify the decrease of H1.3 shown in the Western blot of Fig. 1C, densitometric

quantitation of H1.3 was carried out. The results are expressed as relative arbitrary units (relative intensities) of the ratio of H1.3 to the total H1 fraction of the Coomassie-stained gel of Fig. 1C. As can be seen in the histogram of Fig. 1E, there is a clear decrease in the H1.3 subtype in the sample from the elderly donor. In contrast, the densitometric analysis of the ratio of H1.5 (blot) to the total H1 fraction (Coomassie-stained gel of Fig. 1C) does not show age-related changes (Fig. 1E).

Lastly, we also observed an age-dependent decrease in the ratio of the H1 histones to the core histones (Fig. 1B), which was quantitated by densitometric analysis of the ratio of the total H1 fraction to the total core histone fraction from the Coomassie-stained gel of Fig. 1B (histogram of Fig. 1F). This observation is in agreement with the results of Mitsui et al. (1980).

3.2. CZE analysis of the subtype constitution and phosphorylation state of H1 histones from PBL

To analyse changes in the histone H1 somatic subtype profile and their phosphorylation states, the H1 histones from the lymphocytes of donors from the four different age groups were analysed using CZE. With this method it is possible to analytically separate the homologous members of the H1 family as well as their phosphorylated forms (Kratzmeier et al., 1999; Kratzmeier et al., 2000). The H1 subtypes have a high degree of sequence homology (Ponte et al., 1998). Moreover, they have very similar molecular weights (around 30 kDa in SDS-PAGE, see Fig. 1A) and thus can not be separated by conventional SDS-PAGE. In Figure 2B representative CZE electropherograms from each age group are shown. CZE analysis of PCA-extracted H1 histones revealed 7 main peaks (Fig. 2A/B). The most obvious changes, when comparing the young versus elderly samples, were a significant relative increase of peak 2, a smaller increase in peak 1 as well as a significant decrease of peak 3 and a decrease of peak 6. To determine the identity of the different peaks, mixing experiments (Fig. 2C) were performed with recombinantly expressed H1 subtypes (see Materials and Methods, 2.6, for details). Using this method, the migration times and thus the positions of the unmodified forms of the individual H1 subtypes in the electropherogram of the

H1 histones from the lymphocytes could clearly be assigned, which were: H1.1 (in peak no. 6), H1.2 (in peak no. 5), H1.3 (in peak no. 4), H1.4 (in peak no. 2) and H1.5 (in peak no. 1). Quantitation of the relative area under each CZE peak is shown in Table 1.

In continuation, we wanted to confirm that the observed changes of the CZE peaks can be attributed to the H1 histones and their modifications were not caused by other acid-soluble proteins which were co-extracted with the H1 histones visible in the Coomassie gel (Fig. 2A). For example, it is known that HMG 17 has a similar migration time in the CZE as H1.3 (Kratzmeier et al., 1999). To address this point, PCA-extracted proteins of lymphocytes from four different young and mid-aged donors were pooled and subjected to gel filtration (Fig. 3A). As shown in Figure 3A, the H1 histones, mainly present in fraction 7 and 8, were clearly separated from the HMGs which could be detected in fractions 11 to 13. The first pure H1-containing fraction no. 7 was subjected to CZE. Comparison of the pooled H1 histones before gel filtration and of the pure H1 histone-containing gel filtration fraction no. 7 revealed that no difference in the age-related changing peaks could be observed. Only the H1.3-containing peak no. 4 was found to slightly decrease after the purification of the H1 total histone fraction by gel filtration. Quantitation of the relative area under peak no. 4 before and after gel filtration showed that this decrease was approximately 5% (calculated by quantitation of the relative areas of peaks 1 to 7, data not shown).

Comparison of the CZE pattern obtained from the H1 histones of the lymphocytes with the known pattern of HL60 cells (Kratzmeier et al., 1999, 2000) indicated that peak 3 which had shown a major decrease with age (Fig 2B) represents mono-phosphorylated H1.4 and H1.5 and peak 6 the mono-phosphorylated form of H1.2 (Fig. 4A). The restricted amounts of lymphocytic H1 histones did not allow their separation by reversed-phase HPLC so as to analyse the phosphorylation state of the individual subtypes. To overcome this restriction, we used an indirect approach. The H1 histones, H1.4 and H1.5, from a cycling culture of HL60 cells were separated by reversed-phase HPLC (Fig. 4B). Equal amounts of the different phosphorylated forms of H1.4 and H1.5 were mixed and analysed by CZE (Fig. 4C). Addition of this mixture to lymphocytic histones from a senior donor resulted in the increase of peak 3

in relation to peak 4 (H1.3) (Fig. 4D). From this result we concluded that peak no. 3 can in fact be attributed to the mono-phosphorylated forms of H1.4 and H1.5. Thus, an age-dependent dephosphorylation of these H1 subtypes could be detected (Fig. 2B and Table 1).

3.3. Cell cycle distribution and HP1 α content of PBL

The phosphorylation of H1 histones has till recently been mainly correlated with the cell cycle (Bradbury 1973; 1974). On the other hand, decrease and arrest of cell cycle progression has been shown to be a characteristic property of ageing and senescence, respectively, in the in vitro model ageing cell system of human fibroblasts (e.g., Hayflick and Moorhead, 1961; Howard, 1996). For these reasons, the cell cycle distribution of the lymphocytes from different donors of the four age groups were analysed using flow cytometry as well as by determination of the presence of the S-phase-dependent cyclin A (Fig. 5A/B). A decrease of the cells in S-phase as well of the cyclin A content could be observed in the mid-aged samples compared with those from the young donors. This decrease was also found in the senior and elderly samples (Fig. 5 A/B). Thus, the decrease of cell cycle progression correlated with the observed age-dependent H1 dephosphorylation (Fig. 2; Fig. 5). However, since this decrease in cell cycle progression was found to be at similar levels amongst the samples from mid-aged, senior and elderly donors, while the H1 dephosphorylation of H1.4 and H1.5 (peak no. 3) was observed to be maximal in the senior and elderly age groups and to a much lesser extent in the mid-aged group (Fig 2B), the age-dependent H1 dephosphorylation can not be solely correlated to the observed decrease in cell cycle progression per se.

In this respect, it is noteworthy to mention that a shift was observed in the DNA of only the elderly samples to lower channels, as compared to the DNA flow cytometric profile of the other three age groups, which may be indicative of increased chromatin condensation (Fig. 5A, elderly sample). In light of the fact that Narita et al., (2003) found an accumulation of senescent-associated heterochromatin formations or foci (SAHF) in senescent fibroblasts which are enriched in heterochromatin proteins (HP1), and also that Lezhava

(2001) found that lymphocytes from individuals of 70 years and over, exhibit intensive heterochromatinization, we analyzed the HP1 content in total cell lysates of lymphocytes from donors of all four age groups using an antibody specific for HP1 α . As can be seen from Figure 5C, there is a clear increase in the amount of HP1 α in the elderly sample, not seen in any of the other three age groups.

3.4. Phosphorylation of H2A.X(S139) increases with age

To further confirm that the in vivo / in vitro system of PBL, used in this investigation, indeed reflects age-dependent chromatin alterations, we wished to ascertain by Western analysis the levels of phosphorylated H2A.X (pH2A.X(S139)) in PBL from donors of the four age groups. H2A.X is phosphorylated at serine 139 in response to DNA damage (double strand breaks), recognizes the damaged DNA and is localized where DNA repair is to be initiated (Rogakou et al., 1998; Thiriet and Hayes, 2005). There is ample evidence that DNA double strand breaks accumulate in human cells during ageing and senescence (e.g., Ksiazek et al., 2007; Gorbunova and Seluanov, 2005; Sedelnikova et al., 2004). It has been reported that short telomeres are recognised as DNA breaks resulting in a localisation of phosphorylated H2A.X(S139) at these sites (Hao et al., 2004). Moreover, it has also been found that phosphorylated H2A.X accumulates in these DNA damage foci during ageing in both non telomeric as well as telomeric DNA regions (Sedelnikova et al., 2004; Ksiazek et al., 2007; von Zglinicki et al., 2005). Since DNA double strand breaks in non telomeric regions as well as telomeric regions, have both been shown to increase with age and that there is an accumulation of phosphorylated H2A.X to both types of foci, phosphorylated H2A.X is an epigenetic modification which increases with age and as such can be considered as a marker of ageing and senescence. Indeed, as can be observed in Figure 6, whereas in the young and mid-aged donors pH2A.X(S139) is hardly detectable, in the senior donors there is a significant increase in pH2A.X(S139) which increases even more dramatically in the elderly donors.

4. Discussion

In this study we analyzed the H1 subtype profile and the phosphorylation state of H1 subtypes during the in vivo ageing of human peripheral blood lymphocytes as a function of donor age. The H1 somatic subtypes and their phosphorylation states have been previously analyzed in many cell/tissue systems with respect to growth and development (Lennox and Cohen, 1983; 1984; 1988; Pina et al., 1987) and differentiation (e.g., reviewed in Khochbin, 2001) but, to date, no study has directly addressed their relationship with the in vivo ageing process. We have identified the H1 somatic subtype profile and their phosphorylation states in human lymphocytes and have found differences in the phosphorylation state of two H1 somatic subtypes as a function of in vivo ageing.

PHA-activated lymphocytes were used in this study because it is known that upon antigenic stimulation there are alterations in chromatin organization. Thus we wished to investigate the H1 histone subtype changes and changes in their phosphorylation state that may take place in stimulated cells with respect to age and which may be the result of their age-related degree of heterochromatinization (see discussion below).

Human peripheral blood lymphocytes consist of a large number of T cell subsets. However, there are many studies which have investigated the ageing process using peripheral blood mononuclear cells (PBMC, or the total PBL fraction) which are comprised mostly of T cells (Goronzy et al., 2006). It is known that both CD4 and CD8 cell populations change during ageing. The major characteristic of this immune ageing process is the decrease in the number of naïve T cells with age (Goronzy et al., 2007; Pfister et al., 2006; Kovaïou et al., 2005). The repeated stimulation of memory cells leads to their replicative exhaustion and senescence. However naïve T cells also enter a state of senescence due to their extended replicative history so as to maintain the homeostasis of the naïve compartment during ageing (Nikolich-Zugich, 2005; Goronzy et al., 2007). Moreover, there are numerous studies that have shown that telomere shortening occurs in both naïve (Pfister et al., 2006) and

memory cells (reviewed in Goronzy et al., 2006). Indeed, there is experimental evidence showing that after the age of 65-70 years, an accurate phenotypic distinction amongst naïve and memory cells can not be made (Goronzy et al., 2007; Kovaïou and Grubeck-Loebenstein, 2006). The results of Goronzy et al., (2007) and others (Hakim et al., 2005) indicate that there are discrete time points that mark specific events in immune ageing with an extensive breakdown occurring after the 7th decade of life. This may in fact, parallel the extensive increase in the heterochromatinization of lymphocytes from donors aged 70 years and over found by Lezhava (2001). Our approach to study chromatin changes during ageing of the general lymphocyte population and not a specific population is supported by the fact that indeed all lymphocytic functional compartments undergo extensive age-related failure after the ages of 70-80 years in parallel with a loss of a phenotypically clear distinction amongst the dwindling naïve and compensatory memory cell functions. Thus the results presented in this investigation reflect the global changes that occur in the chromatin histone H1 constitution of the entire ageing lymphocyte population which can be further compared to other ageing cell/tissue systems.

Separation of the PCA-extracted H1 fraction was accomplished by CZE and mixing experiments with recombinantly expressed H1 subtypes were used to determine the identity of the main peaks. Five of the seven main peaks were clearly assigned to contain unmodified forms of the following H1 subtypes: H1.5 (in peak no. 1), H1.4 (in peak no. 2), H1.3 (in peak no. 4), H1.2 (in peak no. 5) and H1.1 (in peak no. 6) (Fig. 2B/C). The most obvious change was a significant age-related decrease in peak no. 3. Since this peak was not one of the unmodified H1 subtypes and it is known that H1.4 and H1.5 are two subtypes that are phosphorylated to a greater extent than the other subtypes (Parseghian and Hamkalo, 2001), we examined the possibility that this peak may be comprised of the phosphorylated forms of these two subtypes. Mixing experiments from reversed-phase HPLC-separated phosphorylated forms of H1.4 and H1.5 from HL60 cells revealed that peak no. 3 contains the mono-phosphorylated forms of H1.5 and H1.4 (pH1.5, pH1.4, Fig. 4). Thus, a distinct dephosphorylation of these two subtypes occurs in lymphocytes as a function of increasing donor age. More

specifically, CZE analyses (see Fig. 2 and Table 1) showed a statistically significant increase in the unmodified form of H1.4 (young: 14.6 ± 1.34 vs. elderly: 30.0 ± 7.36 , $p=0.03$) and a smaller increase in the unmodified form of H1.5 (young: 8.80 ± 2.05 vs. elderly: 14.0 ± 3.05 , $p=0.48$) which however was not found to be statistically significant. These data indicate that the increase in the unmodified forms of these two subtypes (peaks nos. 1 and 2) may be due to the decrease of peak no. 3 (mono-pH1.4 and mono-pH1.5; young: 27.0 ± 2.74 vs. elderly: 15.0 ± 6.54 , $p=0.05$) and may be the result of the dephosphorylation of these two subtypes and most probably not due to de novo synthesis.

Another age-related change found in this study is a decrease of peak no. 6 (young: 4.2 ± 2.05 vs. elderly: 1.04 ± 1.13 , $p=0.01$). Though the mixing experiment with the recombinant H1.1 subtype have shown that this peak contains the H1.1, Western analysis did not detect this subtype in the lymphocytes of any age group. Since the H1 phosphorylated forms have in CZE a higher retention time than their unmodified forms, the age-related decrease of peak no. 6 may also represent dephosphorylation of other subtypes (for example, the monophosphorylated form of H1.2) as was shown in the CZE analysis of the H1 fraction from HL60 cells by Kratzmeier et al. (2000) and perhaps may also consist of the higher phosphorylated forms of H1.4 and H1.5 (2pH1.4, 2pH1.5).

CZE analyses separate the H1 subtypes and their phosphorylated forms so that their relative amounts can be measured. On the other hand, with Western blotting using antibodies specific for each H1 subtype, both unmodified and modified forms of a specific H1 subtype are detected in one band (total amount of each subtype). Western blot analysis showed that H1.3 decreased in the elderly group as compared to the other three age groups. However, CZE analysis did not show a decrease of peak no. 4 (unmodified H1.3) with age. The most likely explanation for this difference amongst the two analyses may be that the age dependent decrease in the synthesis of H1.3 could not be detected by CZE if there was also a change in the modification status of this subtype, most probably dephosphorylation, which resulted in a concomitant increase in the unmodified form of this subtype.

The dephosphorylation of the H1 subtypes, H1.4 and H1.5, found as a function of increasing age can not be attributed solely to cell cycle-related events or cell cycle progression since FACS analysis and cyclin A levels showed different kinetics compared with the dephosphorylation of H1.5 and H1.4. FACS analysis showed a slight, but approximately equal decrease in the proportion of S phase cells in the mid-aged, senior and elderly age groups. Western analysis of cyclin A also showed a decrease in the mid, senior and elderly age groups. However H1.5 and H1.4 dephosphorylation was found to be greatest only in the senior and elderly age groups and not in the mid-aged group.

Moreover, we analyzed the pH2A.X(S139) content of lymphocytes from donors of the four age groups. H2A.X is phosphorylated at serine 139 in response to DNA damage (DNA double strand breaks) and there is an age-dependent increase in pH2A.X(S139) in both non telomeric and telomeric DNA regions (Sedelnikova et al., 2004; Ksiazek et al., 2007; von Zglinicki et al., 2005). In this respect, pH2A.X may be considered an epigenetic modification which increases during ageing and as such may be used as a marker of senescence. In line with this rationale, we found a significant increase in pH2A.X in the senior and more so in the elderly age group (Fig. 6).

It has been postulated for some time that during the process of ageing there is a rearrangement of eu- and heterochromatin domains which alters gene expression in such a manner that irreversibly leads to the senescent phenotype. While this has been shown to be true at the level of gene expression, i.e., certain cell cycle proliferative genes must shut down whereas other senescent-related genes must be activated (Narita et al., 2003), the manner in which these chromatin structural changes occur has been controversial. There is now increasing experimental evidence supporting an increase in heterochromatin (silent chromatin) during the ageing process (Narita et al., 2003; Lezhava, 2001). This results in a gradual shutting down of non-essential cellular processes to the bare minimum, for cellular economization and survival by maintenance of the senescent phenotype. Recent results have shown that there is a formation of senescent-associated heterochromatin foci (SAHF) which are enriched in heterochromatin proteins (HP1) (Narita et al., 2003). Moreover, Lezhava (2001) evaluated the degree of

heterochromatinization (degree of decondensed to condensed chromatin or euchromatin to heterochromatin) of unstimulated and PHA-stimulated lymphocytes from donors of different ages and found age-related differences in heterochromatin and heterochromatinization, irrespective of the stimulated or non stimulated state of the lymphocytes. In fact, Lezhava (2001) found that lymphocytes from individuals of 70 years and over exhibit intensive heterochromatinization. In agreement with these observations, our results showed an increase in the heterochromatin protein, HP1 α , in the lymphocytes of aged individuals (over 70 years old). This finding reinforces the contention that lymphocytes from aged donors may have increased facultative (induced and not constitutive) heterochromatin which increases during senescence. What may be the relationship of our findings to the increase in heterochromatin believed to occur during ageing by the results from Narita et al. (2003), Lezhava (2001) and others (Bandyopadhyay et al., 2007; Herbig et al., 2006; Jeyapalan et al., 2007; Imai and Kitano, 1998) and also indicated from the increase in HP1 α in the lymphocytes of the elderly group observed in this investigation? As described in the Introduction section of this work, recent in vitro and in vivo results support that dephosphorylation of H1 is associated with the maintenance of the H1 subtypes on chromatin or the close association of the H1 subtypes on chromatin and the formation of, or stabilization of condensed structures (Roth and Allis, 1992; Dou et al., 2006). On the other hand, phosphorylation of H1 during the interphase of the cell cycle increases the rate of disassociation of H1 from chromatin and destabilizes the closed structure of chromatin (Contreras et al., 2003; Alexandrow and Hamlin, 2005). There is now experimental evidence that phosphorylation and dephosphorylation of H1, decondenses and condenses chromatin, respectively, either locally or on a more global scale. The proposed mechanism by which this occurs is directly associated with the biophysical properties of DNA. The unphosphorylated H1 tails shield the negative charge of the DNA backbone to allow for chromatin fiber interactions within condensed structures. Upon phosphorylation, the negative phosphate groups weaken the interaction of the tails with DNA to allow for local decondensation (Roth and Allis, 1992; Dou and Gorovsky, 2000; Contreras et al., 2003). Indeed it has been shown that increased H1 phosphorylation results in a more

relaxed chromatin structure and increased accessibility to chromatin-modifying enzymes (Dou et al., 2002; Horn et al., 2002). In line with the above, the dephosphorylation of H1.5 and H1.4 and the decrease of H1.3, in conjunction with the increase in HP1 α found to occur in lymphocytes of the elderly age groups may be related to increased chromatin condensation and the formation of facultative heterochromatin during ageing.

In conclusion, based on our findings and the work of others cited herein, the dephosphorylation of the H1 subtypes, H1.4 and H1.5, found in this study, may be associated with heterochromatin formation and the in vivo ageing process of human peripheral blood lymphocytes. Near future work in this area will show whether the H1 subtype profile and its modification status shows similar or additional age-related changes in other ageing cell/tissue systems as well.

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Table 1
Relative area of each CZE peak

PEAK NO.	YOUNG	MID-AGED	SENIOR	ELDERLY	<i>p</i> -values for the Young-Elderly Pair Groups
1	8.80±2.05 ^a	10.0±2.24	14.0±4.27	14.0±3.05 ^a	^a <i>p</i> =0.48
2	14.6±1.34 ^b	18.8±3.48	30.0±6.88	30.0±7.36 ^b	^b <i>p</i> =0.03
3	27.0±2.74 ^c	25.2±5.22	15.0±4.64	15.0±6.54 ^c	^c <i>p</i> =0.05,
4	15.2±1.64 ^d	16.5±4.27	17.7±1.1	15.8±2.17 ^d	^d <i>p</i> =0.32,
5	24.8±5.02 ^e	24.0±3.81	22.8±3.03	24.4±3.63 ^e	^e <i>p</i> =0.45
6	4.2±2.05 ^f	1.5±1.0	0.9±0.42	1.04±1.13 ^f	^f <i>p</i> =0.01
7	4.8±0.84 ^g	6.2±2.49	3.6±1.67	4.1±2.13 ^g	^g <i>p</i> =0.21

Quantitation of the relative area of each peak after CZE separation of the H1 fraction obtained from lymphocytes from each age group. Values represent the means from 5 donors per age group. ± = S.D. One-tailed Student's t-test was used for statistical analysis. A *p* value of ≤ 0.05 was considered to be statistically different. *P*-values for each peak for the young-elderly donor age pairs are presented in column 6 of this table. (see also Discussion).

Figure legends

Figure 1

Western blot analyses of individual H1 subtypes of lymphocytes from donors of the four different age groups. (A) Specificity of the used H1 subtype-specific antibodies. Recombinantly expressed H1 histones were subjected to SDS-PAGE and stained with Coomassie Blue or used for Western blot analysis using specific antibodies for the detection of H1.1, H1.3 and H1.5 (all purchased from Abcam; see 2.7). Specificity of the antisera used for the detection of H1x and H1.2/H1.5 (Ab4112) was shown in Happel et al. (2005) and Stoldt et al. (2007), respectively. (B) Sulphuric acid-extracted total histone fraction from lymphocytes of donors of the four different age groups. The amounts of total histones that were loaded onto the gel are shown by Coomassie staining. Immunoblot analysis was carried out using antibodies specific for H1.2/H1.5, H1.3, H1.5, H1x and the core histone H3 (used as an additional reference loading control). (C) PCA-extracted H1 histones from lymphocytes from three different age groups. Immunoblot analysis was first carried out for H1.5. The membrane was then stripped and reprobed for detection of H1.3. Loading was monitored by the H1 Coomassie-stained histones. (D) Densitometric quantitation of the relative intensities of H1.2 and H1.3 of Fig. 1B. Results are expressed as relative arbitrary units of the intensities of the ratios of H1.2 (blot of Fig. 1B) to the total core histone fraction of the Coomassie-stained gel of Fig. 1B and also of H1.3 (blot) to the total core histone fraction of the Coomassie-stained gel of Fig. 1B. (E) Densitometric quantitation of the relative intensities of H1.3 and H1.5 of Fig. 1C. Results are expressed as relative arbitrary units of the intensities of the ratios of H1.3 (blot of Fig. 1C) to the total H1 fraction of the Coomassie-stained gel of Fig. 1C and also of H1.5 (blot) to the total H1 fraction of the Coomassie-stained gel of Fig. 1C. (F) Densitometric quantitation of the relative intensities of the ratio of the total H1 fraction to the total core histone fraction of the Coomassie-stained gel of Fig. 1B (expressed as relative arbitrary units). The age groups are: Y, young; M, mid-aged; S, senior and E, elderly donors.

Figure 2

CZE separation of H1 histones from lymphocytes of the four different age groups. H1 histones were prepared by PCA extraction as described under “Experimental procedures”. (A) Aliquots of the extracted histones were subjected to SDS-PAGE, and then gels were stained with Coomassie Blue. (B) Other aliquots were subjected to CZE. Absorbance data at 200 nm were continuously recorded and revealed seven main peaks. Representative CZE electropherograms from each age group are shown. (C) To identify the different subtypes within the CZE pattern, recombinantly expressed H1 subtypes (see 2.6 of Materials and Methods) were mixed with the H1 fraction extracted from lymphocytes of mid-aged donors and subjected to CZE. Five recombinant H1 subtypes could be assigned to peaks in the CZE pattern of the extracted H1 fraction. Y, young; M, mid-aged; S, senior and E, elderly donors.

Figure 3

Removal of acid-soluble non-histone proteins from the H1 fraction by gel filtration. The PCA-extracted H1 histone fractions from lymphocytes from four different young and mid-aged donors (a total of 150 million lymphocytes) were pooled and subjected to P60-gel filtration. (A) Aliquots of the fractions were analysed by Western blotting. The H1-containing fractions were identified by protein staining (Ponceau S) of the blotted membrane (upper part) and HMG17-containing fractions were identified by immunolabelling (lower part). (B) CZE electropherograms of the pooled H1 fractions before gel filtration (Pool) and the pure H1 containing gel filtration fraction number seven (Fraction 7). Peak no. 4, marked with an asterisk, showed a 5% decrease (see text) after gel filtration.

Figure 4

Phosphorylation state of the H1 histones, H1.4 and H1.5, of PBL. (A) Comparison of the CZE pattern of H1 histones from lymphocytes with the known pattern of HL60 cells (Kratzmeier et al., 1999, 2000). (B) Coomassie

staining of HPLC-separated H1 subtypes from HL60 cells. (C) CZE electropherograms of HPLC-separated H1.4, H1.5 and mixed H1.4 and H1.5 from HL60 cells. Unphosphorylated, mono- and diphosphorylated forms of the subtypes are indicated. Phosphorylated forms are labelled as follows: pH1.4 and pH1.5 are the monophosphorylated forms of H1.4 and H1.5, respectively. 2pH1.4 and 2pH1.5 are the diphosphorylated forms of H1.4 and H1.5 respectively. (D) Identification of peak number three of lymphocytic H1 as a mixture of monophosphorylated H1.4 and H1.5. Comparison of the H1 fractions of lymphocytes from young (Y) and senior donors (S). Addition of the mixture of H1.4 and H1.5 histones from HL60 cells to the H1 fraction of the senior age group caused an increase of peak number three in relation to peak number five.

Figure 5

(A) Flow cytometric analysis of the cell cycle distribution of lymphocytes from donors of each age group. Positions (main channel) of the G1 peaks are indicated in brackets. (B) Determination of the relative cyclin A content in total cell lysates from lymphocytes of each age group by Western blot analysis (C) Immunoblot analysis of the heterochromatic protein HP1 α from total cell lysates of lymphocytes from donors of each age group. Core histone H3 was used as a reference loading control.

Figure 6

Western blot analysis of total cell lysates from lymphocytes from donors of the different age groups for phosphorylated H2A.X (pH2A.X(S139)) that is a marker of senescence-dependent DNA damage (double strand breaks). Protein loading was confirmed by staining of the nitrocellulose membrane with Ponceau S.

Fig.1

Fig.2

Figure 3

Fig.4

Figure 5

Figure 6