Mutagenesis vol. 28 no. 3 pp. 307–314, 2013
Advance Access publication 21 February 2013
doi:10.1093/mutage/get004

Protraction of anaphase B in lymphocyte mitosis with ageing: possible contribution to age-related cancer risk

Judith H. Ford*

Centre for Rural Health and Community Development, University of South Australia, 101 Currie Street Adelaide, South Australia 5000, Australia.

*To whom correspondence should be addressed. Tel: + 618 8244 7551; Fax: + 618 8302 5677; Email: judy.ford@unisa.edu.au

Received on November 1, 2012; revised on December 17, 2012; accepted on January 1, 2013

Ageing is associated with a reduction in the fidelity of cell division as shown by increases in trisomic and polyploid cells; however, to date, the underlying age-specific changes in cell division have not been identified. Understanding these specific changes in cell division could give insight into the aetiology some age-related illnesses, especially cancer. Using blood collected from 72 women aged 18–53 years, this study recorded the frequencies of cells in each of the stages of mitosis in synchronised lymphocyte cultures harvested at controlled temperature without microtubule inhibitors. Factor analysis identified four components that accounted for >67.5% of the variance in the data. The component we named 'Spindle elongation efficiency', which was primarily influenced by the time taken to complete anaphase B, showed a major change with age: women aged ≥36 showed a highly statistically significant protraction of anaphase B compared with those aged ≤35 ($t = -2.74$, df = 70, $P = 0.006$) and linear regression showed a logarithmic change in this component with age ($R = 0.297$, $P = 0.011$). This phosphorylation-dependent phase of the cycle is responsible for increasing the distance between the two sets of daughter chromosomes and in older subjects the daughter nuclei at telophase were often poorly separated. Inefficient spindle elongation with ageing probably results from decreased cellular energy. Insufficient force at anaphase B might fail to resolve merotelic kinetochore attachments such that lagging at anaphase would be uncorrected and lead to trisomy and polyploidy in daughter cells.

Introduction

Ageing is a continuous process throughout the life of an organism; however, studies of the biology of ageing are mostly focussed on degenerative diseases and cancer. Nevertheless, it is well known that in women, significant changes in reproductive ageing are observed from the mid-thirties (1,2) when women are still regarded as young and this suggests that some critical aspects of ageing might be occurring at this relatively early age.

Cell division is greatly influenced by ageing. *In vitro,* most cells have only a limited capacity to divide and when they reach that limit, they become senescent (3). *In vivo*, cells that cease to divide may be either senescent, quiescent or terminally differentiated (4) and *in vivo* cellular senescence is highly relevant to ageing and the diseases associated with ageing (5). It has been well established that with *in vitro* ageing, there is a loss of fidelity of cell division and aneuploidy is increased in all stages of cell culture in individuals with premature ageing syndromes (6). Understanding the mechanisms of error and discovering the age at which the rate of error accelerates could have major implications for the prevention of many age-related diseases and disabilities.

Detailed genetic and microscopical studies of the cell cycle have been undertaken in many organisms and have led to the identification of key genes and checkpoints that control the fidelity of cell division (7). Anaphase A, wherein chromosomes move to the spindle poles through the generation of complex forces has been the major focus of many studies. However, the spindle elongation that occurs in anaphase B, which is especially sensitive to cellular ATP (8), may be particularly relevant in human ageing because of the well-substantiated decline of mitochondrial function with ageing.

In human cells, errors of cell division have most commonly been evaluated by karyotyping or by counting micronuclei using the cytokinesis block technique (9). Studies of micronuclei do show age-related changes, but to date, apart from changes in the inactive X chromosome in females, no age-specific cytogenetic mechanism has been identified (10). Moreover, in lymphocytes isolated from both newborns and adult females, >70% of the micronuclei scored contained an X chromosome (11). The cytokinesis block technique also has the other serious limitation that cytokinesis itself cannot be examined and the technique can reduce the detection of laggards and micronuclei (12).

Our earlier karyotypic analysis of lymphocytes from women aged between 20 and 50 showed that changes in the types of chromosome errors seen in lymphocytes (trisomies compared with monosomies) occurred at ~age 36 (13). Our observation was that the rates at which specific human autosomes were involved in aneuploidy (gain or loss) was the same at all ages; however, the ratio of chromosome loss to gain changed dramatically in the late-thirties age group with gain, i.e. trisomy becoming much more common with ageing. In younger women, there were approximately 19 chromosome losses (monosomies) to every gain (trisomy), whereas older women had only about five losses to every gain. We described error as having two phases: (i) initial recruitment, which is chromosome specific and consistent at all ages and (ii) processing, which determines the likelihood of the errant chromosome being lost from the cell or retained by one of the daughter nuclei. Recent data support that 'processing', sometimes referred to as trisomy rescue, is significantly decreased with age, particularly with respect to the Y chromosome in males and the X chromosome in females (14). Because trisomic cells have much higher viability than monosomic cells (15) and contribute more to pathology, it seems that understanding the mechanism underlying this change might give some insight into the cause of increased age-related risks in many diseases, especially cancer.

This article describes the results obtained from a study of all stages of mitosis in human female lymphocytes in an unselected population of 72 women aged between 18 and 53. Detailed statistical analysis has been used to detect the relationships between the different stages of cell division. The data have been interpreted in the light of the molecular genetics and cell biology of the cell cycle.

Materials and methods

Prior to the commencement of this study, intensive laboratory methodological quality assurance was undertaken to ensure that the procedures produced repeatable results.

Subjects

The subjects included 72 apparently healthy women of age range 18–53, whose blood was taken for cytogenetic investigations and who consented to be part of this study.

Lymphocyte cell cycle: culture and harvesting technique

Using aseptic techniques, duplicate cultures without antibiotics were set up for each person. The culture medium was made up of 100 ml of Roswell Park Memorial Institute (RPMI) 1640 medium without L-glutamine (CSL Biosciences), 1.8 ml phytohaemagglutinin (PHA M form, Life Technologies), 1 ml glutamine, 0.2 ml heparin and 20 ml foetal bovine serum (each from CSL Biosciences). All foetal bovine serum samples were batch tested before purchase.

First, 5-ml cultures were initiated (0.5 ml whole blood in 4.5 ml culture medium) and placed in a 5% CO_2 incubator at 37°C. At 72 h after culture initiation, cultures were harvested; never more than six cultures at a time.

Most of the medium was removed with the use of a Pasteur pipette, taking care that the cells were not disturbed. Three ml of pre-warmed (37°C) 0.075 M potassium chloride (KCl) was then added to each of the cultures for 5 min, after which 2 ml of cold fixative (4°C, 3:1 methanol to acetic acid) was rapidly squirted into the hypotonic mix and the cultures placed immediately into a –20°C freezer. After ≥10 min in the freezer, 5 ml of fresh cold fixative was added and then the cells were completely resuspended before centrifugation in 10-ml centrifuge tubes.

Further fixation, spreading and drying were performed in a standard manner and the slides were stained for 3 min in 5% Giemsa stain in phosphate buffer (pH 6.8).

Scoring lymphocyte cell cycle slides

Dividing cells (~100) were scored for each subject. To ensure that every dividing cell was categorised, slides were scanned in a methodical manner, without selection, making sure that no dividing cells were overlooked. The slide coordinates of all abnormal cells were recorded and the cells classified by at least two analysts.

The cells were identified as belonging to one of the following:

Prophase–prometaphase. These two stages were combined because of the difficulty in recognising a clear transition between them (Figure 1a). Cells included in this group include chromosomes that are recognisable but are very long and not yet aligned. When chromosomes attach to the spindle, they become flexed (16). In some cells, at prometaphase, some degree of spindle attachment can be observed.

Ring metaphase. Cells at metaphase should form a perfect ring structure. Quite frequently, some apparently non-attached (displaced) chromosomes will be observed within the ring (Figures 1b and c).

C-metaphase. This is the commonly observed arrested metaphase that is seen when spindles are depolymerised by the addition of colcemid or colchicine (Figure 1h). Here, cells are spontaneously arrested in the c-metaphase and would not have undergone any further division.

Anaphase A. The two groups of chromosomes have clearly moved apart but are still quite close (Figure 1d). This stage can be difficult to recognise because the two separating groups of chromosomes can assume several different conformations when viewed in two dimensions. Cells with obvious lagging chromosomes were noted.

Anaphase B. The chromosome sets should be well separated. A normal separation is one that is ≥30% of the diameter of the anaphase ring (Figures 1e and f). Cells with obvious lagging chromosomes were recorded along with cells where the distance between the two chromosome groups was reduced, as in Figure 1f.

Telophase. The nuclear membranes have reformed (Figure 1g), but the chromatin can still be clearly recognised. Any telophase configurations with micronuclei were recorded.

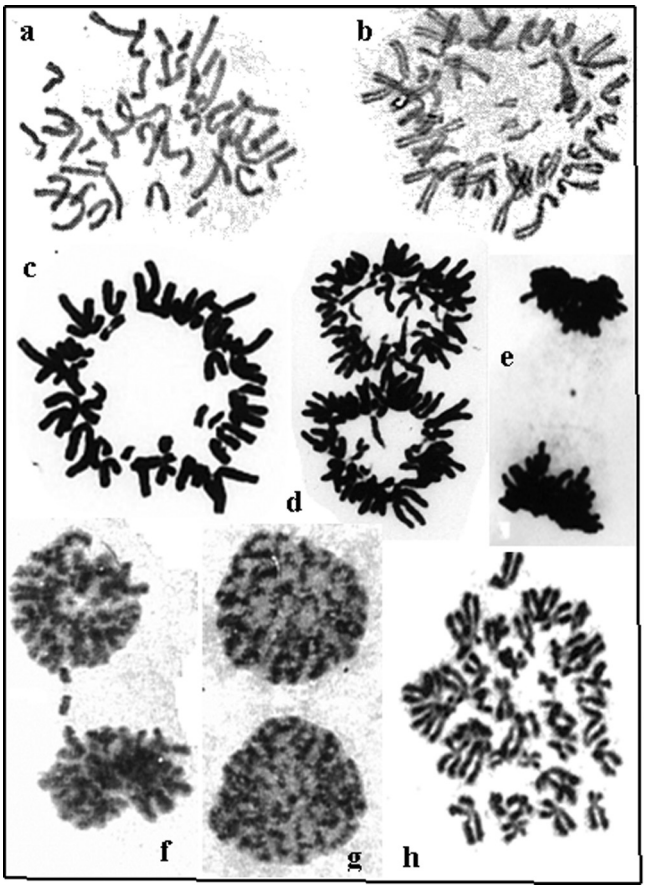

Fig. 1. Typical cells observed in a cell cycle preparation. (**a**) Typical prometaphase with some chromosomes showing flexion; (**b**) early ring metaphase in which some chromosomes are still not attached to the spindle; (**c**) ring metaphase with fully flexed and contracted chromosomes; (**d**) anaphase A showing the two daughter sets of chromosomes retaining their rings; (**e**) late anaphase B in a cell that has achieved full elongation. There appears to be a fragment in the central region of the spindle. (**f**) Late anaphase B with reduced elongation and showing a lagging chromosome; (**g**) telophase cells where there has been a little separation of the two daughter cells; (**h**) C-metaphase showing typical arrest with very few flexed chromosomes.

Statistical analysis

Results were entered into the statistical programme PASW (formerly SPSS) and all data were corrected to an equivalent of 100 cells. The data were analysed to discover any relationships between the different components of the data and to discover whether the age of the women influenced the data.

Data were analysed by Pearson correlation, factor analysis with varimax rotation, unpaired Student's *t*-tests with linear regression and analysis of variance (ANOVA). Factor analysis is of great benefit in determining the underlying relationships between the different variables in a data set and varimax rotation yields results that make it as easy as possible to identify each variable in a single factor.

Results

The subjects were 72 apparently healthy women, whose age range was 18–53, with a mean of 34.66 ± 6.55 and median of 36.00 years. Cells were observed in all stages of mitosis and were classified into the different mitotic stages as depicted in Figure 1.

Slides were examined methodically so as not to exclude any cells. Where possible, 100 dividing cells were classified per subject; however, in some subjects, insufficient cells could be found, whereas in others, it was not appropriate to stop the analysis at

the point where exactly 100 cells had been classified because this would have involved selection. Exactly 100 cells were classified in 43 subjects. Between 91 and 99 cells were analysed in five subjects and 101–123 cells in a further 24 subjects. All values were adjusted to 100 and the adjusted descriptive statistics for cells in each category for all subjects are shown in Table I. Table I shows the normal cells, which are by far the most common cell types and Table II shows the four abnormal cell types, which include c-metaphases, cells with lagging chromosomes at anaphase A, cells with lagging chromosomes at anaphase B and cells with one or more micronuclei (MN) at telophase.

Ring metaphases are the most prevalent cell type, with a mean of 34.81, followed by prophase–prometaphase with a mean of 30.22. Total anaphase cells had a mean of 25.23, with anaphase A cells being slightly more prevalent overall than anaphase B. Telophase was the least frequent stage, with a mean of 6.97.

For each analysis, the proportion of cells seen in the different stages is interpreted as reflecting the relative time spent in the different stages.

The mean number of abnormal cells per subject was 4.77, with lagging at anaphase B at 1.93 being slightly more prevalent than c-metaphases at 1.76. Most observations of lagging at anaphase B were associated with visibly reduced separation of the two chromatid groups; however, reduced separation was relatively common and only a small proportion of these showed lagging. Reduced separation was not scored as a variable because the distance between the chromosome sets formed a more or less continuous range and was probably influenced by slide preparation.

Factor analysis—examination of the relationships between the stages of division

Complex interactions within the various stages of cell division were reflected in these data by many different (negative and positive) statistically significant correlations. To uncover the mechanistic relationships between the variables, factor analysis with varimax rotation was undertaken. The results are shown in Tables III and IV: four major components account for >67.5% of the total variance.

The four components account for 18.8, 18.8, 15.4 and 14.6% of the variance respectively (a total of 67.6%). Thus, the first two components are of equal and high importance and the other two are each of slightly lesser importance. Each of these four

Table I. Descriptive statistics—normal cells per 100 cells analysed

Statistic	Prophase + prometaphase	Ring metaphase	Anaphase A	Anaphase B	Telophase
Mean	30.22	34.81	13.34	11.89	6.97
Standard deviation	6.76	8.19	4.54	4.85	4.25
Standard error of mean	0.79	0.96	0.53	0.57	0.50
Median	29.17	33.66	12.50	11.11	6.78
Mode	27.27	29.41[a]	12.50[a]	14.14	4.04
Minimum	15.46	19.63	2.20	2.04	0.00
Maximum	45.00	59.79	23.47	23.23	22.45

[a]Multiple modes exist. The smallest value is shown.

Table II. Descriptive statistics—abnormal cells per 100 cells analysed

Statistic	C-metaphase	Lagging anaphase A	Lagging anaphase B	Micronuclei at telophase
Mean	1.76	0.30	1.93	0.78
Standard deviation	2.19	0.64	2.10	1.24
Standard error of mean	0.26	0.07	0.25	0.15
Median	1.03	0.00	1.01	0.00
Mode	0.00	0.00	0.00	0.00
Minimum	0.00	0.00	0.00	0.00
Maximum	6.00	2.70	10.81	6.00

Table III. Principal components identified by varimax rotation

Rotated component matrix

	Component			
	Spindle assembly checkpoint	Spindle elongation efficiency	Compromised microtubule assembly	Cytokinesis completion
Prometaphase	0.789	–0.343	–0.131	–0.106
Ring metaphase	–0.919	–0.102	–0.154	–0.172
Anaphase A	0.127	–0.676	–0.029	–0.098
Anaphase B	0.037	0.802	0.053	–0.183
Telophase	0.239	0.288	0.080	0.713
Telophase with micronuclei	–0.043	0.550	–0.350	0.238
C-metaphase	0.319	0.180	0.749	–0.238
Anaphase A with lagging	–0.199	–0.170	0.806	0.211
Anaphase B with lagging	–0.095	–0.129	–0.062	0.749

In each component the variables showing high correlations are highlighted. The highlighted variables make the major contribution(s) to the component and were used to define the component as explained in the text.

Table IV. Variance explained by major components

Component	Initial eigenvalues			Extraction sums of squared loadings			Rotation sums of squared loadings		
	Total	% Variance	Cumulative %	Total	% Variance	Cumulative %	Total	% Variance	Cumulative %
1	1.837	20.406	20.406	1.837	20.406	20.406	1.693	18.812	18.812
2	1.661	18.452	38.858	1.661	18.452	38.858	1.692	18.804	37.616
3	1.348	14.982	53.840	1.348	14.982	53.840	1.386	15.405	53.021
4	1.236	13.735	67.575	1.236	13.735	67.575	1.310	14.554	67.575
5	0.924	10.268	77.843	–	–	–	–	–	–
6	0.832	0.246	87.089	–	–	–	–	–	–
7	0.632	7.019	94.108	–	–	–	–	–	–
8	0.522	5.805	99.913	–	–	–	–	–	–
9	0.008	0.087	100.000	–	–	–	–	–	–

major components is characterised by two major variables that are highly correlated, with range between 0.676 and 0.919. The major function represented by each component was interpreted from the cell cycle literature and names were assigned to reflect these: namely 'Spindle assembly checkpoint' (Factor 1), 'Spindle elongation efficiency' (Factor 2), 'Compromised microtubule assembly' (Factor 3) and 'Cytokinesis completion' (Factor 4).

Component 1 has been named Spindle assembly checkpoint (17): cells do not move from prophase to metaphase until the spindle is fully assembled and all chromosomal kinetochores are properly attached to the spindle (18). The inverse relationship observed in this component between the frequency of cells in prophase–prometaphase and the frequency of cells seen in ring metaphase reflects the rate at which full spindle assembly is completed. An increase in prophase–prometaphase relative to ring metaphase can be interpreted as a delay in spindle assembly.

Component 2 has been interpreted as Spindle elongation efficiency. In this component, there is an inverse relationship between the frequencies of cells observed in the two stages of anaphase. The transition from anaphase A to anaphase B involves a change in the function of the microtubules from pulling the chromosomes apart to increasing the distance between the two chromatid groups through spindle elongation (19). Spindle elongation is dependent on phosphorylation, probably of kinesin-5 (20,21). If phosphorylation is compromised, then spindle elongation will be less efficient and the distance between the two chromatid sets at telophase will be reduced. Inefficiency of elongation increases the time spent in anaphase B and thus increases the proportion of cells in anaphase B relative to anaphase A.

The abnormal variable 'Telophase with micronuclei' also shows a strong association with the Spindle elongation efficiency component (value = 0.550) and has a negative association with Compromised microtubule assembly (value = –0.350).

Component 3 does not include any of the normal phases of division but only anaphase A cells that have lagging chromosomes and c-metaphase cells. This component has been given the name Compromised microtubule assembly.

Component 4 includes normal telophase cells and anaphase B cells with lagging chromosomes and has been named 'Cytokinesis completion'. Phosphorylation of mitotic kinesin-like protein 2 is required for cytokinesis (22). Cells remain in telophase until cytokinesis is completed; thus, a higher proportion of telophase cells reflects a delay in completing cytokinesis. The variable 'lagging at anaphase B' and the variable telophase are highly correlated and this implies that cellular conditions associated with lagging at anaphase B also predispose to delay in completing cytokinesis.

Student's t-tests—examination of the stages of division and principal components by age

Unpaired Student's *t*-tests were used to compare the frequency of each of the different cell types and the values of the four factors between older and younger women. The cut-off point for the analysis was chosen as age 36, both because this is the approximate age at which reproductive incompetence accelerates (1,2) and because it is also the group median.

The results for women aged <36 years were compared with those ≥36 and the results of the Student's *t*-tests are shown in Table V. Comparison of the means between women aged <36 and those ≥36 demonstrated that of the four components, only Spindle elongation efficiency showed a significant age difference. This was quite marked: $t = -2.74$, degrees of freedom (df) = 70, and $P = 0.008$. It is clear from the data that the difference is due to an increase in the time spent in anaphase B in older subjects: the mean number of cells in anaphase B was 10.33 for the younger women and 13.39 in the older women: $t = -2.84$, df = 70, and $P = 0.006$.

The presence of micronuclei at telophase is also associated with this component and the number of cells with micronuclei (albeit small) was significantly increased in the older women (1.06 compared with 0.48), $t = -2.34$, df = 70, and $P = 0.022$.

Linear regression—examination of the principal components by age

Linear regression was conducted for each of the four components by age. Only the second component, Spindle elongation efficiency, showed a statistically significant change with age and this was best described by a logarithmic model. The results are shown in Table VI. There is a significant increase in the Spindle elongation inefficiency component with age ($F = 6.793$, $P = 0.011$). The correlation coefficient R is 0.297 and accounts for 8.8% of the variation in the data. Figure 2 is a graphical representation of this relationship with the line of best fit.

Discussion

Identification of key components in mitosis in cultures of human lymphocytes

Lymphocyte cultures offer the advantage of synchronisation. In PHA-stimulated cultures grown at controlled pH and temperature, the first wave of metaphases usually occurs at 48 h and the second at 72 h. Here, 72-h cultures were used because although some synchronisation is lost, the number of dividing cells is much higher. By harvesting the cultures with strict temperature control, without microtubule depolymerisation and with rapid fixation at

Table V. Results of *t*-tests for comparison of findings in women aged younger or older than age 36

Component	Age group	*N*	Mean	Standard deviationD	Student's *t*-test	df	Significance
Spindle assembly checkpoint	<36	35	0.07	1.06	0.62	70	NS
	≥36	37	–0.07	0.95	–	–	–
Spindle elongation inefficiency	<36	35	–0.34	0.98	–2.74	70	$P = 0.008$
	≥36	37	0.23	0.98	–	–	–
Compromised microtubule assembly	<36	35	–0.11	0.82	–0.95	70	NS
	≥36	37	0.11	1.15	–	–	–
Cytokinesis checkpoint	<36	35	–0.11	0.90	–0.90	70	NS
	≥36	37	0.10	1.09	–	–	–
Prophase & prometaphase	<36	35	31.07	6.80	1.20	70	NS
	≥36	37	29.17	6.67	–	–	–
Ring metaphase	<36	35	35.29	8.64	0.62	70	NS
	≥36	37	34.32	7.82	–	–	–
Anaphase A	<36	35	13.83	4.45	1.10	70	NS
	≥36	37	12.66	4.61	–	–	–
Anaphase B	<36	35	10.33	4.36	–2.84	70	$P = 0.006$
	≥36	37	13.39	4.96	–	–	–
Telophase	<36	35	6.94	4.64	–0.14	70	NS
	≥36	37	7.08	3.91	–	–	–
Telophase with micronuclei	<36	35	0.48	1.28	–2.34	70	$P = 0.022$
	≥36	37	1.06	1.19	–	–	–
C-metaphase	<36	35	1.82	2.18	0.22	70	NS
	≥36	37	1.71	2.23	–	–	–
Anaphase A with lagging	<36	35	0.16	0.43	–	–	–
	≥36	37	0.42	0.77	–1.73	56.7	NS
Anaphase B with lagging	<36	35	1.69	2.00	–0.86	70	NS
	≥36	37	2.11	2.20	–	–	–

NS, not significant.
The components showing statistically significant differences between the younger and older women are highlighted.

Table VI. Logarithmic regression model of age by spindle elongation efficiency

Model summary				
R	*R*-square		Adjusted *R*-square	Standard error of the estimate
0.297	0.088		0.075	0.962
Coefficients				
	Unstandardised coefficients		Standardised coefficients	
	B	Standard error	Beta	*t*	Significance
ln(Age)	1.492	0.573	0.297	2.606	0.011
(Constant)	–5.265	2.023	–	–2.602	0.011
ANOVA					
	Sum of squares	df	Mean square	*F*	Significance
Regression	6.281	1	6.281	6.793	0.011
Residual	64.719	70	0.925	–	–
Total	71.000	71	–	–	–

–20°C, we were able to study the numbers of cells at the different stages of mitosis. Using factor analysis to identify the major components, it was possible to interpret these observations in terms of some of the known cell cycle molecular mechanisms.

Age-dependent and -independent errors of division

Within the age group studied, this study showed no evidence of an age-related change in any of the stages of cell division up to the end of anaphase A. There is evidence of error occurring in these early stages, but these are not influenced by ageing. Cells that are delayed in the transition from prometaphase to ring metaphase are presumed to have problems with either kinetochore attachment to the spindle or problems with microtubule polymerisation. Cells in which polymerisation fails are detected as c-metaphases, but cells where one or a few chromosomes fail to attach to the spindle frequently progress past the spindle checkpoint. Such unattached, or possibly mono-oriented chromosomes, are observed in ring metaphases as 'displaced' chromosomes and we have previously demonstrated that apart from the late-replicating X chromosome, other chromosomes are displaced as a function of their size (23); small chromosomes are displaced most frequently.

With the exception of 'Telophase with micronuclei', none of the other three error types was significantly increased with ageing and factor analysis gives some insight into their aetiology. Both 'c-metaphase' and 'lagging' at anaphase A are highly correlated with each other but with no other variables that we measured. This has been interpreted as reflecting compromised microtubule assembly because c-metaphases are routinely induced for karyotypic analysis by agents such as colcemid and colchicine that prevent microtubule polymerisation.

Lagging at anaphase B is 'not' correlated with lagging at anaphase A nor with prolongation of anaphase B or microtubule assembly. It is, however, strongly associated with

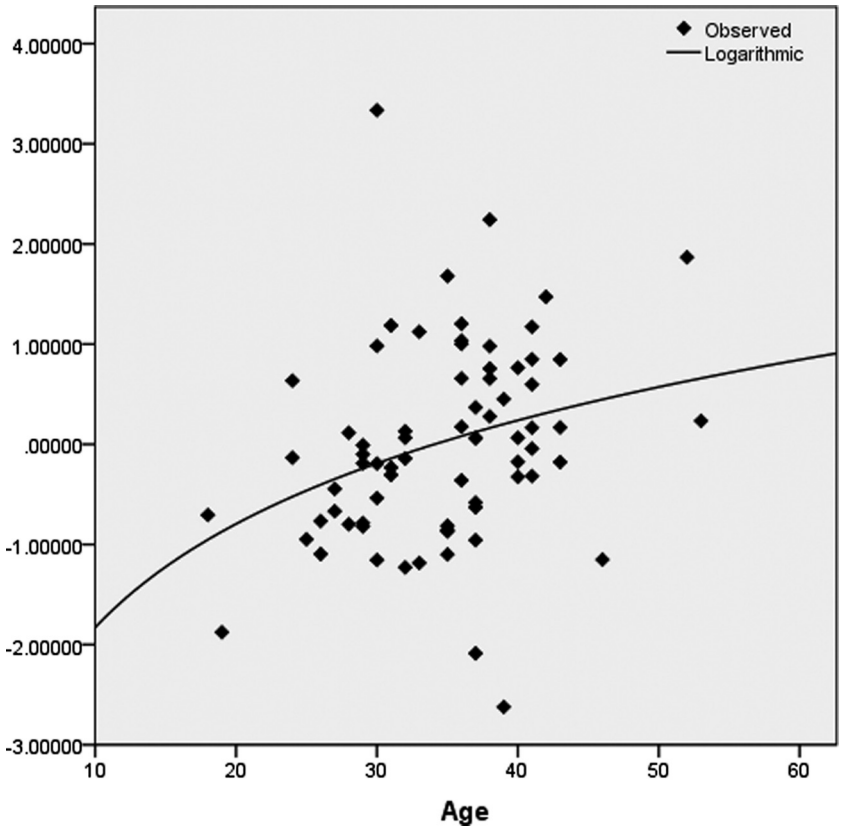

Fig. 2. Graph of the regression model of age (*x* axis) by scores for spindle elongation efficiency. Individual scores are shown together with the logarithmic line of best fit. Note that with age, scores moves from 'negative', where anaphase A is more prevalent than anaphase B, to the 'positive' reverse situation. The change from negative to positive occurs in about the mid-thirties age group although there is considerable individual variation.

prolongation of telophase. It is likely that the anaphase-B laggards have merotelic kinetochore attachments. Chromosomes with merotelic attachments (one of the kinetochores attached to both spindle poles and the other to a single pole) are not detected by the spindle checkpoint and so proceed to anaphase B. Anaphase-B lagging was increased with ageing but not significantly so.

Merotelic kinetochore attachment has been considered in detail in the recent literature and is proposed as the primary mechanism of chromosome instability in cancer cells (24). If, like yeast, correction of merotelic attachments in human lymphocytes relies on forces that are exerted during spindle elongation (25), this could explain why many of the cells with lagging at anaphase B were observed to have reduced separation of the daughter nuclei. Because this was not an analysis of live cells, we could not observe the fate of the lagging chromosomes. However, if it is assumed that reduced separation of daughter nuclei reflects lack of force during spindle elongation, then it seems likely that many of these lagging chromosomes would not be corrected and could segregate into daughter cells leading to trisomies.

The correlation of lagging at anaphase B with the prolongation of telophase is explained by recent work (26) that shows that even subtle reduction in *Polo-like kinase* (*Plk1*) gene activity can produce anaphase dysfunction and prolong telophase. There are several key genes involved in chromosome segregation at anaphase and phosphorylation is critical to their activity. *Plk1* is a gene that has major functions in vertebrate cell division, especially in the stages of spindle elongation and cytokinesis. *Plk1* plays a role in coordinating chromosome segregation and cytokinesis (27). *Plk1* seems to be involved in the initiation of anaphase B and when anaphase is complete, *Plk1* localises to the mid-zone, where it is involved in the formation of the contractile ring.

Telophase cells with micronuclei chromosomes seem to be a further independent class of abnormal cells. These cells are significantly increased with ageing ($P = 0.02$) and are correlated with protracted anaphase B (0.550 in rotated component matrix). A recent study of MN formation was conducted using live-cell imaging in HeLa cells (28). One mechanism (named 'Mechanism 2' by the authors) contributed to 63.66% of the MN whose origin was traceable. The chromosomes that went on to form the MN either moved more slowly than the other chromosomes or remained at the cell equator during anaphase–telophase. Some of these laggards were observed to decondense to form MN at the next interphase. These authors found, however, that only 164/610 observed lagging chromosomes went on to form MN and that the remainder appeared to disappear, usually before the completion of chromosome decondensation.

Because telophase cells with MN are not correlated with anaphase-B lagging in our material, we think that they probably arise by a mechanism that is separate and different from merotelic attachment. It is possible that Mechanism 2 (mentioned in the previous paragraph) includes both this unknown mechanism and merotelic attachments and that most of the latter are either corrected or the chromatids segregate erroneously. In studies that identify the specific chromosomes in human MN, most are found to contain inactive X chromosomes (11), but in

some cases, a single MN might contain more than one chromosome (14). The loss of the inactive X chromosome is known to increase with female ageing (29), but it seems that this mechanism is independent of other age-dependent errors of division and specifically leads to MN formation. Some chromosomes that appear to disappear in the HeLa study and chromosomes that lag in anaphase B cells in which the spindle fails to elongate might move into the daughter telophase cells rather than form MN.

Defective phosphorylation—the underlying age-related defect in cell division?

This analysis demonstrates that even from the relatively early age of 36, there is a highly significant increase in the proportion of anaphase B cells observed in the lymphocyte cultures. In younger women, anaphase A cells are more prevalent but anaphase B cells become more prevalent after age 36. Our regression model (Figure 2) based on these data indicates that ageing is strongly associated with increased time spent in anaphase B and by implication, decreased efficiency of anaphase B.

Age-related changes in mitochondrial function are central to most models of ageing (30) and age-related defects in mitochondria have been demonstrated by electron microscopy (31) and bio-energetic function studies (32,33). The decline in oxidative phosphorylation in ageing tissues has been demonstrated in a plethora of studies, particularly in isolated mitochondria (30) as well as in mitogen-stimulated murine T lymphocytes (34). Anaphase B but not anaphase A is dependent on ATP and is inhibited by ATP inhibitors (35).

Examination of specific mitochondrial function in human lymphocytes with ageing, i.e. subjects with a mean age of 35 compared with those with a mean age of 80 (36), showed significant declines only in mitochondrial complex II and complex III functions. Functions of complexes I and IV were not affected by age. T lymphocytes are unusual among normal cells in using glycolysis and glutaminolysis as their primary energy pathways (37). This is especially relevant to the age-related changes in complex II because complex II links directly to the tricarboxylic acid cycle, coupling succinate oxidation to ubiquinol reduction in the mitochondrial membrane. Because reduced nicotinamide adenine dinucleotide (NADH) and complex I are bypassed (38,39), the age-related reduction in complex II is very likely to underlie the significant effect of age on anaphase B. Because the decline in complex II is progressive, we predict that any future studies that might extend our observations in older subjects would show a continuous decline in the efficiency of anaphase B.

Reduced efficiency of anaphase-B spindle elongation: relevance to age-related cancer?

Our previous work showed that in the mid-thirties, chromosome error in women led to trisomy rather than monosomy as in younger women (13) and at the time, we referred to this difference as a difference in processing errant chromosomes. We now propose that the lack of separation of the two daughter cells, which accelerates in the mid-thirties age group, changes the fate of chromosomes that are poorly attached to the spindle such that in younger women, poorly or incorrectly attached chromosomes are likely to be eliminated from both daughter cells, whereas in older women, the chromosomes are likely to be included in one of the daughter cells. Reduction in the distance between the two chromosome sets was also given as the explanation for why cytochalasin B-treated lymphocytes showed ~49% lower frequencies of lagging chromosomes/chromatids and MN after treatment with low concentrations of colchicine than untreated cells (12). The findings were explained by assuming that in cytochalasin B-blocked cells, there is a shorter distance between the poles and that this leads to (i) lagging chromosomes being engulfed in the nearest daughter nuclei and (ii) insufficient distance between the two daughter nuclei, leading to the formation of polyploid nuclei.

Trisomy is the most common chromosome abnormality found in cancers of all types (40) and it is well known that the risk of cancers of most types increases with ageing. This work has been performed in lymphocytes, but if similar age-related reductions in the efficiency of the stages of cell division that depend on phosphorylation occur in all tissues, then this defect could be a major contributor to the age-related risk of cancer.

Men and women

This study has been conducted in women because more women than men were referred to our laboratory during the period in which the study was undertaken. Moreover, our previous studies were also undertaken in women and so it was convenient to compare the results of cultures studied with this cell cycle analysis with those undertaken with karyotyping. In future studies, it would be interesting to both extend the age range and include both male and female subjects, as well as studying a wide range of cell types.

Acknowledgements

I would like to thank Maryam Hor for her expert technical assistance with both the cell cycle cultures and analysis of the slides.

Conflict of interest statement: None declared.

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