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INVITED PAPER

CYTOGENETIC METHODS FOR BIODOSIMETRY AND RISK INDIVIDUALISATION AFTER EXPOSURE TO IONISING RADIATION

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Measurement of dicentric chromosomes in human lymphocytes has been applied to assess dose received by potentially overexposed people and estimate risk for health effects. Since the dicentrics in exposed people decrease with time, the introduction of fluorescent *in situ* hybridisation enables to measure stable translocations for biodosimetry and address old or long-term exposures. In addition, premature chromosome condensation, which enables analysis in interphase, offers several advantages for biodosimetry. However, dose and risk estimates derived using cytogenetics and adequate calibration curves are based on the assumption that all individuals respond equally to radiation. Since increased radiosensitivity has been associated with cancer proneness, there is particular interest for risk assessment at the individual level. Towards this end, the efficiency of dynamics that govern DNA repair and apoptosis, as well as the conserved cellular processes that have evolved to facilitate DNA damage recognition using signal transduction pathways to activate cell cycle arrest and preserve genomic integrity, are being investigated. Recent work in cancer cytogenetics and on the modulation of radiation effects at the chromosome level using changes in gene expression associated with proteins or factors such as caffeine or amifostine treatment during G_2 to M-phase transition, reconfirmed the importance of G_2 chekpoint in determining radiosensitivity and of the cdk1/cyclin-B activity in the conversion of DNA damage into chromatid breaks. G_2-chromosomal radiosensitivity may offer, therefore, a basis for the identification or testing of key genetic targets for modulation of radiation effects and the establishment of a screening method to detect intrinsic radiosensitivity.

INTRODUCTION

In case of exposure or suspected overexposure to ionising radiations, it is important to assess quickly and as precisely as possible the doses received by the exposed or potentially overexposed individuals in order to adopt the best medical arrangement and obtain risk estimates in a prospective way[1–3]. For many years, the measurement of chromosomal aberrations and rearrangements in human peripheral blood lymphocytes (PBL) has been used as biological dosimeter for the estimation of absorbed doses in real or suspected radiation exposures (Figure 1). Specifically, the analysis of dicentrics, rings, micronuclei and excess chromosome fragments using conventional cytogenetics and premature chromosome condensation (PCC) that enables analysis of chromosomal aberrations in interphase, have been extensively applied for this purpose. Furthermore, since the yield of unstable aberrations decreases with time in exposed people, the introduction of fluorescent *in situ* hybridisation (FISH) made it possible to measure more persistent type of damage, e.g. stable translocations for biodosimetry in order to address old or long-term exposures[4,5]. Dose and risk estimates derived using cytogenetic methods and adequate calibration curves, however, are based on the assumption that all individuals respond equally to radiation, but this is not always true and at present, a particular interest of biological dosimetry has been to establish a methodology to indicate inter-individual sensitivity of people to ionising radiation. Since increased radiosensitivity has been highly associated with cancer proneness, there is in addition particular interest in elucidating the mechanisms underlying genetic susceptibility to radiation sensitivity. Towards this end, the efficiency of dynamics that govern cell cycle arrest, DNA repair and apoptosis, as well as the conserved cellular processes that have evolved to facilitate DNA damage recognition using signal transduction pathways to activate cell cycle arrest and preserve genomic integrity, are being investigated. The assay of choice for studies of radiosensitivity is the cell cycle based G_2-chromosomal radiosensitivity assay[6]. Recent work on the modulation of radiation effects at the chromosome level using changes in gene expression associated with proteins or factors such as caffeine[7] or amifostine treatment[8] during G_2 to M-phase transition of normal and AT cells, showed the importance of cdk1/cyclin-B activity for the conversion of DNA damage into chromosomal damage and provided direct evidence that G_2-checkpoint facilitates repair of chromosomal damage. In view of the potential importance of these observations, G_2-chromosomal radiosensitivity may offer a basis for the identification

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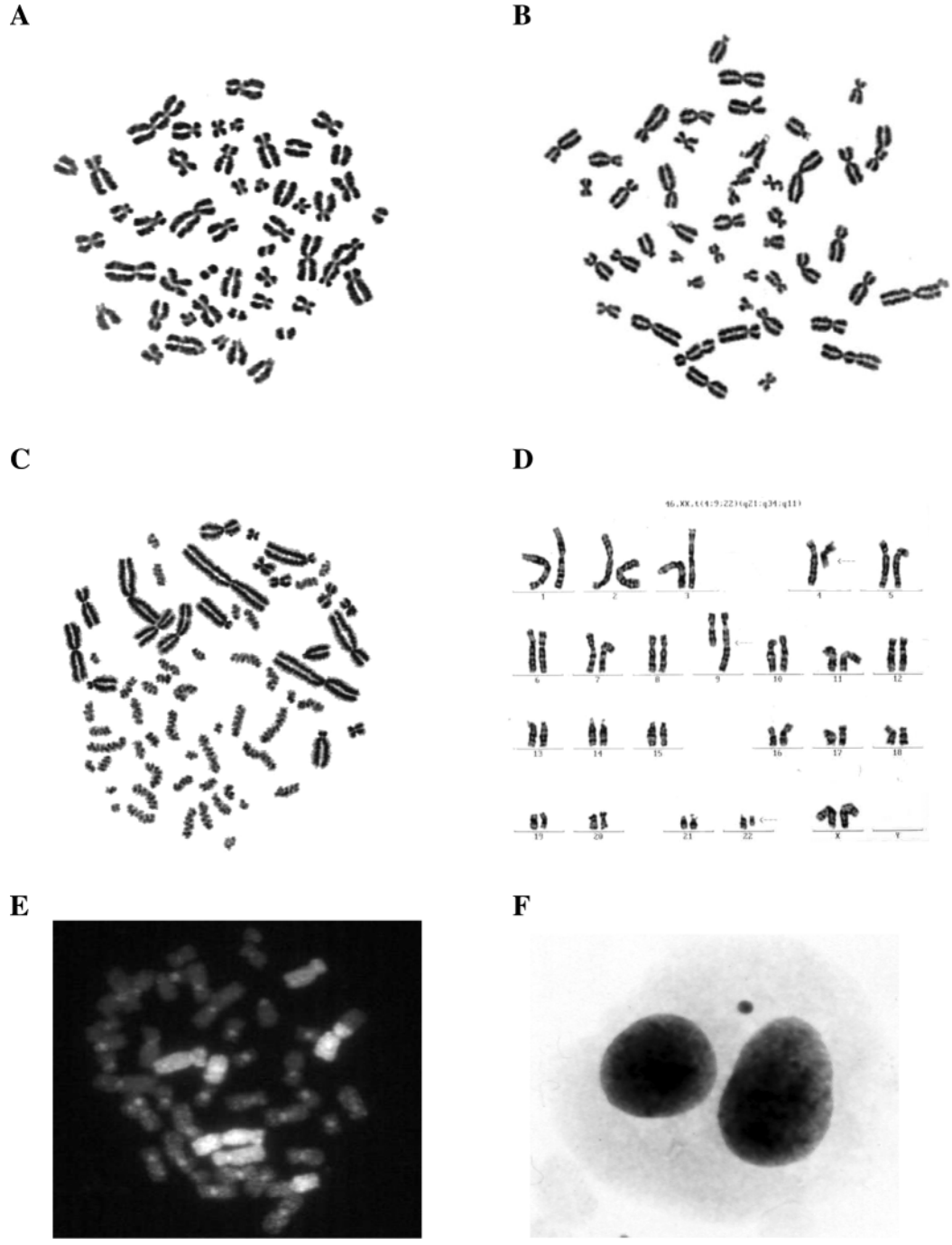

Figure 1. The basic cytogenetic endpoints used as bio-indicators for absorbed dose estimation and risk assessment at the individual level: (A) conventional analysis of dicentrics at metaphase. (B) Analysis of chromatid breaks at metaphase to indicate intrinsic G_2-chromosomal radiosensitivity. (C) Analysis of prematurely condensed lymphocyte chromosomes that enables absorbed dose estimates within two hours after exposure. (D) GTG-banding and karyotyping to identify clonal reciprocal translocations for risk individualization. (E) FISH analysis to detect reciprocal translocations for old or long-term exposures. (F) Scoring of micronuclei for a quick absorbed dose estimation.

or testing of key genetic targets for modulation of radiation effects, and the establishment of a screening method to detect intrinsic radiosensitivity that would facilitate the risk assessment at the individual level.

MATERIALS AND METHODS

Cell culture and irradiation conditions

Three EBV-transformed lymphoblastoid cell lines (LCL), GM 15786, GM03188A and GM09899 derived from an AT patient, an obligatory ATM heterozygote and a normal individual, respectively, were used. Cells were maintained in RPMI (Gibco) supplemented with Hepes and sodium bicarbonate, 15% fetal bovine serum (FBS), 1% L-glutamine (2 mM) and antibiotics (penicillin: 100 U ml^{-1}; streptomycin: 100 μg ml^{-1}). All incubations were at 37°C in a humidified incubator, in an atmosphere of 5% CO_2 and 95% air. For culturing PBL, 5 ml of blood was drawn from three clinically characterised AT homozygotes and healthy controls. Whole blood (0.5 ml) was added to 4.5 ml of McCoy's 5A medium supplemented with 10% FBS, 1% phytohaemagglutinin (PHA), 1% glutamine and antibiotics. Cultures were incubated at 37°C for 72 h before use in experiments. Irradiation was carried out in a GammaCell 220 irradiator (Atomic Energy of Canada Ltd, Ottawa, Canada) at room temperature and at a dose rate of 1 Gy min^{-1}. PHA was dissolved in water at a concentration of 0.24 mg ml^{-1}. Caffeine was prepared as a 100 mM stock solution in PBS. All chemical were from Sigma unless stated otherwise.

The G_2-chromosomal radiosensitivity assay

Proliferating cells (lymphoblasts or lymphocytes) were exposed to radiation (0.5 or 1 Gy) and incubated for 30 min at 37°C to allow division of cells irradiated at mitosis. Subsequently, the culture was treated with colcemid for 1 h to arrest dividing cells at metaphase, as described in Ref. (9). At 90 min post irradiation, cells from all cultures were collected by centrifugation, treated in 75 mM KCl for 10 min, fixed in methanol:glacial acetic acid 3:1 (v/v) and processed for cytogenetics analysis. Standard procedures were used for chromosome preparation and staining[10]. Chromosomal damage was visualised and quantified as chromatid breaks in the subsequent metaphase. For each experimental point, approximately 50 cells were scored for chromatid damage based on standard criteria. For scoring, chromatid breaks and gaps were considered; the latter are scored as breaks only when longer than the chromatid width. Light microscopy was coupled to an image analysis system (MetaSystems, Germany) to facilitate scoring. The spontaneous aberration yield was subtracted to obtain the radiation-induced G_2 yield of chromatid breaks. Standard deviations of the mean values from two to three independent experiments were calculated.

Cell fusion and premature chromosome condensation

Cell fusion and PCC induction using polyethylene glycol (PEG) was performed according to Pantelias and Maillie methodology[10]. Briefly, CHO or TK6 mitotic cells and G_0 PBL were washed separately with McCoy's 5A medium without serum and mixed in a ratio of ~1:5 in a 15 ml round-bottom culture tube. After centrifugation at 200 g for 5 min, the supernatant was discarded without disturbing the cell pellet and 0.15 ml of 50% (w/v) PEG (PEG 1500, Boehringer Mannheim) was added all at once and held for ~1 min. Subsequently, 1.5 ml of PBS was slowly added, the tube was gently shaken and the cell suspension was centrifuged at 200 *g* for 5 min. The supernatant was discarded and the pellet resuspended in 0.7 ml of McCoy's 5A growth medium supplemented with colcemid (0.05 ml from a 10-5 M stock solution). After 75 min at 37°C, cell fusion and PCC induction was completed and chromosome preparations were obtained by standard cytogenetic procedures. Routinely, 30–50 lymphocytes in G_0 phase were scored for excess chromosome elements (excess than 46) for each experimental point. Standard deviations of the mean values from three independent experiments were calculated.

RESULTS

G_2-chromosomal radiosensitivity in healthy individuals

Blood samples from 15 healthy donors were tested for their chromosomal radiosensitivity in the G_2 phase of the cell cycle as described in Materials and Methods. The assays were undertaken in duplicates for 0.5 and 1 Gy. The mean spontaneous chromatid breaks were 1 per 100 cells that was subtracted from the yield in irradiated cells to obtain the induced yield of chromatid breaks. The results are shown in Table 1.

Intrinsic G_2-chromosomal radiosensitivity

Three repeated assays for intra- and inter-individual G_2-chromosomal radiosensitivity estimates for donors no. A, F, E, H, K, N showed clear differences in the yield of radiation-induced chromatid breaks after 0.5 or 1 Gy gamma-irradiation. To investigate further whether the induced yield of chromatid breaks as scored at metaphase after irradiation in G_2-phase is intrinsic, experiments with mixed blood cultures between male and female donors were initiated and irradiated at 72 h as described in Materials and Methods. Using this experimental design it was

Table 1. G_2-chromosomal radiosensitivity for 15 healthy donors.

Donor no.	Sex	Induced chromatid breaks per cell (mean ± SD)	
		0.5 Gy	1 Gy
A	M	2.5 ± 0.3	4.5 ± 0.5
B	F	0.8 ± 0.1	1.6 ± 0.2
C	M	0.7 ± 0.1	1.8 ± 0.2
D	F	1.4 ± 0.2	3.3 ± 0.3
E	M	0.6 ± 0.1	1.1 ± 0.1
F	M	1.5 ± 0.2	3.4 ± 0.4
G	F	1.0 ± 0.1	2.5 ± 0.3
H	F	0.9 ± 0.1	2.0 ± 0.2
I	M	0.8 ± 0.1	2.9 ± 0.3
J	F	1.3 ± 0.1	2.9 ± 0.3
K	M	0.9 ± 0.1	2.5 ± 0.3
L	M	0.7 ± 0.1	1.9 ± 0.2
M	M	0.9 ± 0.1	2.3 ± 0.3
N	F	1.5 ± 0.2	4.0 ± 0.5
O	F	1.1 ± 0.1	2.7 ± 0.3

Table 2. The G_2-chromosomal radiosensitivity assay in separate or mixed cultures confirms that inter-individual differences in radiosensitivity are intrinsic and not due to culture conditions.

Donor no.	Sex	Induced chromatid breaks per cell (mean ± SD)			
		Separate cultures		Mixed cultures	
		0.5 Gy	1 Gy	0.5 Gy	1 Gy
A	M	2.7 ± 0.3	4.8 ± 0.5	2.4 ± 0.3	4.5 ± 0.3
D	F	1.5 ± 0.2	3.5 ± 0.3	1.3 ± 0.9	3.4 ± 0.3
E	M	0.7 ± 0.1	1.3 ± 0.1	0.5 ± 0.1	1.1 ± 0.1
H	F	0.8 ± 0.1	2.2 ± 0.2	0.9 ± 0.1	1.9 ± 0.2
K	M	0.8 ± 0.1	2.7 ± 0.3	1.0 ± 0.1	2.5 ± 0.2
N	F	1.7 ± 0.2	4.2 ± 0.5	1.6 ± 0.2	4.3 ± 0.4

ensured that lymphocytes from both male and female blood donors were proliferating and irradiated under exactly the same conditions. The G_2-chromosomal radiosensitivity analysis was easily carried out in chromosome preparations from mixed cultures since male (X,Y) metaphases were easily distinguished from the female (X,X) metaphases. The results are shown in Table 2 and demonstrate that G_2-chromosomal radiosenstitivity is intrinsic, since it is shown that cells exhibit similar radiation sensitivity in the mixed population as they did when irradiated alone. To further verify this observation, the G_2-chromosomal radiosensitivity was studied in different cell lines and primary cells and the results are shown in Table 3.

Table 3. Different cell types demonstrate differences in G_2-chromosomal radiosensitivity.

Cell type	Chromatid breaks per cell (mean ± SD)	
	0.5 Gy	1 Gy
CHO	0.7 ± 0.1	1.6 ± 0.2
XRS-5	2.5 ± 0.3	5.7 ± 0.5
Lymph	1.5 ± 0.2	2.6 ± 0.3
TK6	3.7 ± 0.4	6.8 ± 0.6
MCF-7	4.3 ± 0.4	9.6 ± 0.8
$ATM^{-/-}$ LCL	4.4 ± 0.5	8.2 ± 0.9
$ATM^{+/-}$ LCL	2.8 ± 0.3	4.8 ± 0.5
$ATM^{+/+}$ LCL	1.6 ± 0.2	3.0 ± 0.2
AT Patient A	4.2 ± 0.3	8.1 ± 0.7
AT Patient B	4.4 ± 0.5	8.9 ± 0.8
AT Patient C	4.1 ± 0.4	7.8 ± 0.7

[CHO, XRS-5, TK6 and MCF-7 data are adapted from Terzoudi et al., Int. J. Rad. Biol. 76: 607–615 (2000)]

Table 4. Different yields of chromatid breaks per Gy is obtained when Go lymphocytes are fused to mitotic cells with different cdk1/cyclinB activities.

Dose (Gy)	Excess PCC fragments/cell (mean ± SD)	
	CHO mitotics	TK6 mitotics
0	0	0
1	3 ± 0.2	7 ± 0.6
2	7 ± 0.6	13 ± 1.1
3	11 ± 0.9	22 ± 2.4
4	14 ± 1.2	27 ± 2.5

Increased G_2-chromosomal radiosensitivity and cdk1/cyclin-B activity

The hypothesis that increased G_2-chromosomal radiosensitivity may not only be a consequence of differences in DNA repair capabilities, but it may also be linked to up-regulation of cdk1/cyclin-B activity during G_2 to M-phase transition was tested. Isolated G_0 lymphocytes were irradiated to doses up to 4 Gy and the chromosomal damage was scored in prematurely condensed chromosomes by fusing them to CHO or TK6 mitotic cells that have higher cdk1/cyclin-B activity level than the CHO cells[9]. The results are shown in Table 4.

G_2-chromosomal radiosensitivity and G_2-checkpoint defects

The hypothesis that G2-checkpoint defects in human cells after DNA damage can also dramatically affect

Table 5. Caffeine treatment of normal or AT heterozygotes increases G_2-chromosomal radiosensitivity to the level of AT cells.

Cell line/Donor	Conventional analysis of chromatid breaks at metaphase ± SD	
	1 Gy (mean ± SD)	1 Gy + Caffeine (4 mM) (mean ± SD)
ATM−/− (GM15786)	8.9 ± 0.9	8.5 ± 0.8
ATM+/− (GM03188)	4.0 ± 0.5	9.2 ± 0.9
Control (GM09899)	2.0 ± 0.3	8.9 ± 1.0
Control A	2.8 ± 0.4	9.6 ± 0.9
Control B	3.9 ± 0.5	9.7 ± 0.8

G_2-chromosomal radiosensitivity, was tested with two sets of experiments. In the first set caffeine was employed to abolish G_2 checkpoint by inhibiting ATM[11,12]. Caffeine was administered 30 min before irradiation in G_2 phase and chromatid breaks were scored at the subsequent metaphase. The results are shown in Table 5. In the second set of experiments, elevated temperatures were used to modify the G_2/M transition after irradiation in G_2 phase by culturing the cells immediately after irradiation at 40°C in the presence of colcemid. Under these experimental conditions the mitotic index was increased by 30% and the effect on the yield of chromatid breaks is demonstrated in Figure 2.

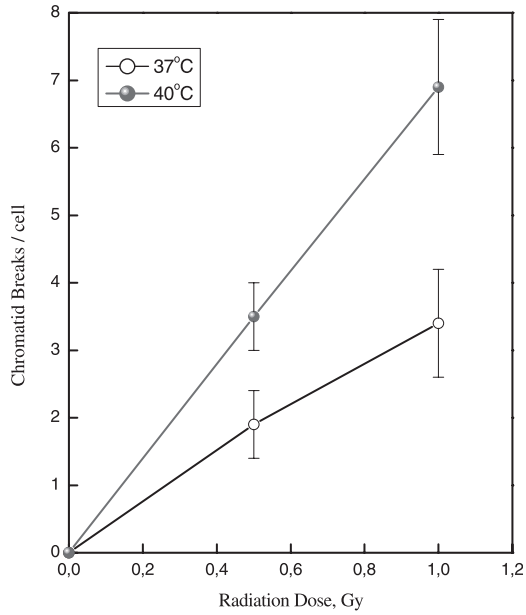

Figure 2. G_2-chromosomal radiosensitivity was increased up to 100% when cells were culturted at 40°C after irradiation [Figure adapted from Terzoudi and Pantelias; Mutagenesis **12**, 271–276 (1997)].

DISCUSSION

Cytogenetic biodosimetry has been applied for long time to obtain absorbed dose estimates in real or suspected overexposures. The basic cytogenetic endpoints used as bio-indicators are shown in Figure 1, and a comparison of the results obtained using these cytogenetic methods in the case of an accidental overexposure has been reported recently[13]. In particular, the measurement of dicentrics has been used for more than 30 y[14] and is considered to be the standard method for biodosimetry[15]. The background level of dicentric chromosomes is very low giving the method a high degree of sensitivity. When scoring a few thousand metaphases the detection level following an acute exposure can be as low as 0.02 Gy[16]. Scoring of micronuclei is easier but the sensitivity is hampered by the inter-individual variability in the spontaneous micronucleus frequency. When scoring 1000 binucleated cells *in vivo* exposure in excess of 0.2 Gy could be detected[17]. For retrospective dosimetry GTG-banding and in particular FISH analysis for stable reciprocal translocations is the method of choice[5]. Scoring of ~1000 metaphases for translocations after single colour FISH allows the detection of 0.3 Gy[18]. PCC, which enables analysis in interphase, offers several advantages for biodosimetry particularly in case of emergency or partial body exposures[19–22].

To supplement the observation of clinical symptoms and physical dose reconstruction, a particular interest of biodosimetry, has been not only to obtain absorbed dose estimates using adequate calibration curves under the assumption that all individuals respond equally to radiation-induced chromosome aberrations, but also to find a way to indicate inter-individual radiosensitivity and describe risk assessment at the individual level[2]. Towards this goal, the use of FISH after an accidental overexposure could not only be applied for dose reconstruction and absorbed dose estimation, but most importantly for detecting clonal cells with stable reciprocal translocations. The detected clonal cells with reciprocal translocations can be then characterised in terms of breaking points and fusion genes and identified by means of G-banding and karyotyping. The fusion genes involved in the observed reciprocal translocations could have a prognostic potential if compared to those that characterise preleukemia and leukemia syndromes and diseases of the hematopoietic tissue.

The links between cellular radiosensitivity and cancer predisposition are well established and arose from investigations into chromosomal instability

syndromes such as ataxia-telangiectasia[23]. Variations in radiosensitivity between individuals may be a consequence of differences in DNA repair capacity due to specific mutations or polymorphisms in DNA repair genes[24] or alternatively, may be linked to cell cycle and feedback control mechanisms during the G_2 to M-phase transition[9]. The first demonstration of a G_2-checkpoint defect in human cells after DNA damage came from observations in cells of patients with the inherited, cancer-prone syndrome, ataxia-telangiectasia[25,26]. It was found that the G_2 arrest after exposure to ionising radiation was much less pronounced in AT than in normal cells and it was suggested that this might account for the enhanced G_2-chromosomal radiosensitivity of AT cells. However, this hypothesis was not easily reconciled with other observations on quantitative relationships between mitotic inhibition, chromosome damage, age and gender[27]. On the other hand, evidence has been reported that unrejoined DNA double strand breaks are not predominantly responsible for chromosomal radiosensitivity of AT cells[28]. The rationale of the present study was to further investigate the role and the importance of the G_2 checkpoint as well as of the cdk1/cyclin-B activity during the G_2 to M-phase transition, in affecting conversion of DNA damage into chromatid breaks and possibly in determining intrinsic G_2-chromosomal radiosensitivity.

The results presented in Table 1 demonstrate a similar range of radiation-induced chromatid aberrations in a group of normal individuals to those reported in the literature, where comparable scoring criteria were used[23]. By performing analysis on chromosome preparations obtained from separate and mixed lymphocyte cultures between male and female donors (Table 2), a very good intrinsic assay reproducibility was demonstrated, by eliminating any variability in culture conditions that might underlay intrinsic inter-individual variation in G_2-chromosomal radiosensitivity among blood donors. These results confirm the findings published in Ref. (23,29). To investigate further this observation, G_2-chromosomal radiosensitivity was examined in different cell types. The results shown in Table 3 demonstrate an intrinsic sensitivity for the different cells used. With respect to the mechanisms underlying such a variation in chromosomal radiosensitivity, it is worth mentioned that Terzoudi *et al.*[9] observed that the higher the cdk1/cyclinB activity level of the cell line tested for G_2-chromosomal radiosensitivity, the higher the yield of chromatid breaks scored at metaphase after a certain dose in G_2 phase. In addition, other investigators have reported that the differential G_2-phase radiosensitivity of human PBL, found in normal individuals using the 'G_2-phase chromosome radiosensitivity assay', could be attributed to heterogeneity in cellular progression to mitosis[30]. Variability in cdk1/cyclinB activity could in fact underlie such heterogeneity. The results shown in Table 4 reconfirm the proposed hypothesis and emphasise the important role played by the cdk1/cyclinB activity level in determining G_2-chromosomal radiosensitivity during G_2 to M-phase transition. The yield of chromatid breaks was analysed directly in interphase lymphocytes after irradiation by means of PCC using mitotic inducer cells that have different cdk1/cyclinB activities. The higher the activity of mitotic PCC inducer cells the higher the yield of chromatid breaks scored, in agreement with the results reported earlier[9,31–33].

To test the hypothesis that G_2-checkpoint defects during the G_2 to M-phase transition can also affect G_2-chromosomal radiosensitivity, caffeine was employed to abolish G_2 checkpoint by inhibiting ATM. The results in Table 5 show that treatment with caffeine of normal or AT heterozygotes increase G_2-chromosomal radiosensitivity to the level of AT cells in agreement to earlier studies[11]. However, caffeine did not increase any further the G_2-chromosomal radiosensitivity of the AT cells. Additional support to the testing hypothesis comes from the experiments in which elevated temperatures were applied to modify the G_2/M transition after irradiation in G_2-phase[33]. When the cells were cultured immediately after irradiation at 40°C in the presence of colcemid, the mitotic index was increased by 30% and the effect on the yield of chromatid breaks and, therefore, the G_2-chromosomal radiosensitivity was increased up to 100%, as shown in Figure 2. The use of caffeine and post-irradiation culture of lymphocytes at 40°C, has enabled us to develop and standardise a new G_2-chromosomal radiosensitivity protocol for the detection of intrinsic radiosensitivity.

In summary, even though inter-individual variations in G_2-chromosomal radiosensitivity may be a consequence of differences in DNA repair capabilities, the data presented in this paper emphasise the role and the importance of cdk1/cyclin-B activity and of the G_2-checkpoint during G_2 to M-phase transition, in affecting conversion of DNA damage into chromatid breaks and in determining possibly chromosomal radiosensitivity in G_2 phase. Elucidation of the mechanisms underlying G_2-chromosomal radiosensitivity may offer a basis for the identification or testing of key genetic targets for modulation of radiation effects and the establishment of a screening method to detect intrinsic individual radiosensitivity. Furthermore, in accidental overexposures the use of cytogenetic methods for intrinsic radiosensitivity and absorbed dose estimation and identification of fusion genes in clonal cells with stable translocations would facilitate the risk assessment at the individual level.

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