The need for dynamic methods for measuring cell cycle perturbations: a study in radiation-treated lymphoblastoid cell lines of varying p53 status

R Gilchrist, M E Lomax and R S Camplejohn

Richard Dumbleby Department of Cancer Research UMDS St Thomas Hospital London UK

(Received 30 July 1998 second revision accepted 11 December 1998)

Abstract. Reports on the p53-related cell cycle and apoptotic responses of EBV-transformed lymphoblastoid cell lines to DNA damage have led to some confusion. This may be due to differences in the nature of the specific *p53* mutations under examination, but it can also be partly attributed to methodological and analytical problems (e.g. the inappropriate use of static DNA histograms for cell cycle analysis). Taking seven lymphoblastoid cell lines derived from both normal individuals and Li-Fraumeni Syndrome/Li-Fraumeni-Like (LFS/LFL) patients of differing p53 status, we completed a detailed study of radiation-induced cell cycle perturbations. Using BrdUrd pulse labelling and flow cytometry it was found that, regardless of p53 status, the cells did not arrest in G₁ despite all of the lines showing p53 upregulation 3 hours postirradiation. The irradiated cells did, however, show a general slowing both in S-phase entry from G₁ and in movement through S-phase. These facts would not have been apparent from the analysis of static DNA histograms. The problems with the use of static methods to assess changes in the dynamics of cell cycle progression apply not only to studies involving EBV-transformed cell lines, but also to a wide range of investigations into the molecular control of cell proliferation.

INTRODUCTION

Rare cancer-prone families were first recognized in the late 1960s (Li & Fraumeni 1969) with a very particular clinical picture involving early onset sarcomas, breast carcinoma, brain tumours, lymphoma and adrenal carcinoma, these families are said to exhibit Li-Fraumeni Syndrome (LFS). More recently families have been recognized, with a similar spectra of malignancies, which do not fully meet the precise criteria of classical LFS. Such families have been called Li-Fraumeni-like (LFL) (Birch *et al* 1994). In 1990 Malkin *et al* demonstrated a relationship between *p53* mutations and LFS. *p53* has clearly been shown to be a tumour suppressor gene and *p53* mutation and deletion are the most common genetic defects seen in sporadic clinical cancer. We are interested in studying the nature of germline defects which can lead to loss of p53 function in LFS/LFL families (Camplejohn *et al* 1995, Lomax *et al*

Correspondence: Dr R S Camplejohn, Richard Dumbleby, Department of Cancer Research, Rayne Institute, St Thomas Hospital, Lambeth Palace Road, London SE1 7EH, UK. E-mail: r.camplejohn@icrf.icnet.uk

1997, Lomax *et al.* 1998). As these families are rare, it would be valuable to establish model systems which enable easy propagation and testing of specific p53-related defects. For such systems to be of use, the functional attributes of the inherent p53-related defects would have to be preserved. EBV-transformed lymphoblastoid cell lines (LCLs) are easily set-up and maintained and retain the donor genotype through long periods of tissue culture (Lalle *et al.* 1995). Unfortunately, both our own data and a series of papers published during the past 3 years show that integration of the virus appears to distort the normal p53-related properties of these cells (Okan *et al.* 1995), bringing their use as a model into doubt. Nevertheless, different studies have revealed conflicting results in LCLs in relation to p53-related cell cycle arrest and apoptosis in response to a variety of DNA damaging agents. Although this may be due partly to the specific nature of the mutations under examination, the way in which the genetic insult is achieved, or to other biological factors, a considerable part of this confusion can be attributed to the use of unsuitable methodology and incomplete data analysis. A particular problem relates to the common practice of using static DNA histograms to infer changes in progression of cells around the cell cycle, particularly when cell cycle perturbations are investigated as a part of molecular biological studies. In the context of LCLs, a number of studies have relied on the use of static methods to demonstrate cell cycle arrests following gamma irradiation (Little *et al.* 1995, Okan *et al.* 1995, Williams *et al.* 1996, Goi *et al.* 1997). Using DNA histograms derived flow cytometrically, Williams *et al.* (1996) reported that all LCLs exhibited a radiation-induced G₁-arrest irrespective of their p53 status. Delia *et al.* (1997) reported some particularly interesting results in that different p53 mutations led to differences in the induction of G₁-arrest in LCLs. Unfortunately, their cell cycle data are based solely on the analysis of static DNA histograms. It is well established that analysis of such static data to interpret dynamic changes in cell cycle progression can be misleading (Quinn & Wright 1990). In this study, cell cycle perturbations in response to gamma radiation have been assessed in seven EBV-transformed LCLs derived from normal and LFS/LFL individuals using dynamic methodology. The main aim of the current study is to establish reliable protocols for the assessment of cell cycle progression delays and apoptosis following gamma irradiation in EBV-transformed LCLs.

MATERIALS AND METHODS

EBV-transformed lymphoblastoid cell lines

Human peripheral blood B-lymphocytes were transformed with Epstein-Barr virus to produce immortal lymphoblastoid cell lines as shown in Table 1. These cell lines were obtained from Nigel Spurr then at ICRF Clare Hall (lines 389, GM0892A, GM0621) and John Boyle at the Paterson Laboratories in Manchester (all other lines). All lines were grown in RPMI supplemented with glutamine and 10–15% heat inactivated fetal calf serum. Cells were seeded at 2×10^5 cells/ml and were not permitted to grow in excess of 7×10^5 cells/ml.

Cell fixation for bromodeoxyuridine (BrdUrd) labelling

The cells were seeded at 2×10^6 cells per T25 flask and allowed to recover overnight. $2 \mu\text{M}$ BrdUrd was added to the flasks for 10 min and then washed out with prewarmed medium. The cells were either irradiated or mock-irradiated and then returned to a 37°C incubator before significant cooling could occur. Fixation at each relevant time point was in ice cold 70% ethanol following a single phosphate-buffered saline (PBS) wash.

Table 1. Results from the seven lymphoblastoid cell lines studied, all of which are from different families. MA196L¹ is from an individual from a Li–Fraumeni family with a stop mutation at codon 136. The donor individual has been found to be negative for this mutation. Cell lines MA109L² and MA13L² have no *p53* mutation as confirmed by whole coding gene sequencing (Varley *et al* 1997). A representative time point of 6 h was chosen to illustrate the reduction in cells in the S-phase compartment throughout the experiment.

Cell line	Derivation and <i>p53</i> status	% Reduction in RM (%)	% Reduction in S-phase entry at 6 h (%)	Potential doubling time unirradiated (h)
GM0892A	Normal volunteer WT/WT	16	24.5	17.3
GM0621	Normal volunteer WT/WT	20.5	45.1	14.2
MA196L ¹	LFS WT/WT	22.2	43.5	15.9
MA109L ²	LFS WT/WT	15.7	26.6	16.9
MA8L	LFS MUT/WT (codon 175)	12	34.2	14.3
MA13L ²	LFL WT/WT	30	36.5	17.7
389	LFL MUT/WT (codon 337)	22.3	37.4	19.0

LCL irradiation procedure

Irradiation was carried out using a Gammacell 1000 Elite (Nordion International) containing a caesium 137 source with a dose rate of 8.58 Gy per minute. Doses of 4 Gy were given as described by Camplejohn *et al* (1995).

Staining of BrdUrd labelled cells

Fixative was spun off and replaced with 1 ml of 0.1 M HCl containing 1 mg/ml pepsin. Following a 12 min incubation in a 37°C water bath, the reaction was halted using PBS. After a further PBS wash, anti-BrdUrd antibody (mouse monoclonal from Becton Dickinson catalogue no. 347580) was added to the cells (20 µl added to 80 ml PBS/0.5% Tween/1% fetal calf serum) which were then left for one hour at room temperature. Following two PBS washes, FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse antibody (Dako Ltd, Cambridge, UK) was added (10 ml added to 90 ml PBS/Tween/serum) for a further 30 min in the dark at room temperature. After a final wash in PBS, the cells were re-suspended in 1 ml propidium iodide/RNase solution (50 mg PI/200 mg RNase per ml of PBS) and allowed to stain for a minimum 10–15 min before analysis.

Analysis of BrdUrd staining

Data from 10⁴ dual stained cells were accumulated on a FACSCalibur flow cytometer (Becton Dickinson UK Ltd, Oxford, UK) and saved in list mode. Single intact nuclei were separated from debris and clumps by gating on an FL2 area/width plot. Cells which had been in S-phase at the start of the experiment were recognized by positive green fluorescence (FITC).

Relative movement (RM)

RM was calculated as reported by Begg *et al* (1985). This method involves the calculation of the mean red (PI) fluorescence of those BrdUrd-labelled cells which have not yet passed through their first postlabelling mitosis. RM was calculated for each experiment from the

slope of a straight line fitted using the least squares method to data obtained between 0 and 6 h after BrdUrd labelling (see Fig. 1).

Entry of unlabelled G₁ cells into the S-phase

At each time point, a histogram of BrdUrd-unlabelled cells was produced by combining the gate on the FL2 area/width plot (a standard procedure to exclude clumps and debris) with a second gate on the FL2 area (representing DNA content)/FL1 (representing BrdUrd labelling) plot so as to include only unlabelled cells. The percentage of unlabelled cells in S-phase was estimated using a trapezoid, five compartment model with Modfit software (Phoenix Flow Systems, CA). This method yields a reliable and largely objective measure of the number of cells leaving the G₁-phase and entering S-phase at various times after real or mock irradiation.

Western blotting for p53 expression

To determine whether p53 was induced following gamma irradiation, cells were followed over a 12 h time period after exposure to a 4 Gy dose. Protein lysates were made in NP-40 lysis buffer and 5 µg of total protein run out on an 8% polyacrylamide gel. D0-1 ascites antibody (D0-1 is a mouse monoclonal antibody which binds to amino acids 20-25 at the amino-terminal end of human p53 protein; this antibody was obtained from Prof David Lane, Dundee, UK) was used as a probe and binding levels detected by chemiluminescence.

RESULTS

Probing of western blots with D0-1 ascites antibody and detection by chemiluminescence confirmed that p53 was upregulated in each cell line in response to DNA damage. This induction occurred within three hours of radiation exposure (data not shown) in all lines regardless of mutation status. Compared to the other six cell lines, the 389 cells exhibited high constitutive levels of p53 which was still capable of being upregulated after 4 Gy radiation. This reflects the behaviour of the donor's p53 protein *in vivo* as reported in Lomax *et al.* (1997). All of the cell lines tested also exhibited high constitutive levels of the antiapoptosis protein bcl-2.

To follow the progress of cells through the cell cycle, they were pulse labelled with BrdUrd and examined over a 6 h time period. This time point was chosen from previous pilot experiments which determined that most labelled cells had progressed through mitosis in the majority of the cell lines by this time. After this point, the movement of cells around the cell cycle becomes more difficult to analyse. Using these data, potential doubling times (T_{pot}) were calculated for all unirradiated cell lines (Table 1). As can be seen T_{pot} varied from just over 14 h up to 19 h. From the BrdUrd/PI staining profiles, the rate of entry of unlabelled G₁ cells into the early part of S-phase and the rate of movement of labelled cells through S-phase (relative movement (RM)) were calculated. In all experiments (14 experiments on seven different cell lines), the RM of the irradiated cells was reduced by between 12% and 30% compared with the unirradiated control cell RM value as shown in Table 1. In Figure 1a raw data are illustrated showing RM for irradiated and unirradiated cells from a single experiment on the 389 cell line. From these data the reduction in the RM following irradiation was calculated for each experiment. For the sake of brevity the mean RM data of four individual experiments on two representative cell lines: GM0621 and 389 is illustrated (Fig. 2a). The RM of the irradiated cells is $79.5\% \pm 8.5\%$ and $77.75\% \pm 3.7\%$ (standard

Figure 1. a, Shows the rate of movement of BrdUrd labelled cells through the S-phase (RM) for irradiated and unirradiated 389 cells during an 8 h period following 4 Gy or mock irradiation. Similar data, also for 389 cells, are given for the entry of unlabelled cells into S-phase for irradiated and unirradiated cells in **b**

Figure 2. Cell cycle analysis on two representative cell lines; GM0621 (WT/WT) and 389 (MUT/WT). In **a**, the mean relative movement values of four BrdUrd labelling experiments are plotted. RM is a numerical figure relating to the rate at which cells move through the S-phase of the cell cycle. In both cases, the irradiated cells are shown to slow down in comparison to the controls. In **b**, the reduction in unlabelled cells which have entered S-phase from G₁ at various times up to 6 h after irradiation is depicted; these results are plotted as the reduction in the percentage of unlabelled cells after radiation compared to unirradiated controls calculated as follows: reduction in unlabelled S-phase cells = $P_R - P_C$ where P_C is the percentage of unlabelled cells in S in the control sample at a particular time and P_R is the percentage in the equivalent irradiated sample.

deviation) of the control cell value, respectively. No trend was visible to indicate a difference between mutant and wild type cell lines. Using the Modfit programme to analyse the unlabelled fraction of cells entering S-phase from G₁, percentage values were calculated for each time point. Figure 1b illustrates the raw data from a single cell line (389) with and without exposure to radiation. Again, in all experiments (14 on seven cell lines) a reduction in the number of cells in the unlabelled S-phase population following radiation was apparent. Representative results on cell lines GM0621 and 389 are plotted in Figure 2b showing the reduction in unlabelled S-phase cells at times up to 6 h after irradiation as a percentage of

control values. A summary of the results for all the cell lines is given in Table 1. Despite the reduction in the number of unlabelled cells in the S-phase compartment, cells did continue to enter the early stages of S-phase indicating that no absolute G_1 arrest occurred. This can be demonstrated in the bivariate FL2 A/FL1H flow cytometry plot (Figure 3) where unlabelled cells are seen entering S-phase. As reported in other cell types, a G_2 block lasting for at least 24 h also occurred in the irradiated cells of all the cell lines tested. This G_2 block is visible in Figure 3 where S-phase cells begin to accumulate in the G_2 phase.

DISCUSSION

LCLs derived from LFS individuals carrying germline *p53* mutations are heterozygous for *p53*, having one wild-type and one mutant allele. The loss of one functional allele can result in changes in DNA damage responses in lymphoid cells. For example, in both heterozygous knockout mice and in individuals carrying germline mutations the apoptotic response to radiation in such cells is significantly reduced (Clarke *et al* 1993, Camplejohn *et al* 1995). This is something incidentally not found in the EBV-transformed cell lines used in this study where all LCLs showed an increase in apoptosis following irradiation irrespective of their *p53* status in the current study (Gilchrist *et al* 1998).

Questions remain concerning whether LCLs in general (i.e. those derived from normal individuals as well as those from mutation carriers) show a G_1 -arrest following irradiation and, if so, whether such a G_1 -arrest can be disrupted by particular heterogeneous *p53* mutations. Williams *et al* (1996) in common with the data in this paper, found no difference in terms of G_1 -arrest between LCLs with and without a heterozygous *p53* mutation. They did, however, suggest that both show a G_1 -arrest after radiation. This conclusion was based on the interpretation of static flow cytometric DNA histograms and their data look very much like that shown in panel C of Fig 3 in this paper. However, in the present study BrdUrd labelling clearly shows that what is occurring is a slowing down in the passage of cells into and through S-phase rather than a complete arrest of cells in G_1 . The cells entering S-phase after irradiation consist of those cells already committed to proliferate at the time of irradiation and those which have passed the decision point after this time. We cannot clearly distinguish between these two categories of cell in our analysis but it is likely that most of the cells measured entering S-phase after irradiation have made the decision to do so following the time of irradiation. There are a number of reasons for believing this, firstly the experiments looking at entry of unlabelled cells into S was carried out over a 6 h period and by 6 h in the unirradiated cells over half of all G_1 cells had entered S and even in the irradiated samples something approaching 50% of cells had entered the S-phase. Thus it is unlikely that all of these cells had already passed the G_1 /S checkpoint prior to irradiation. This impression is confirmed by limited data that we have on some of the cell lines where we actually looked at cell cycle traverse 24 h after radiation. In this case also there is evidence of a slowing of cells leaving G_1 and entering S.

The question as to whether particular *p53* mutations lead to changes in G_1 -arrest after radiation is more difficult to answer as different investigators have looked at cell lines with different mutations and not one of them has used a dynamic method to measure cell cycle progression except in the present study. For example, Delia *et al* (1997) reported that a heterozygous mutation at codon 282 in the *p53* gene led to G_1 -arrest in an LCL following irradiation, whereas cells with a 286 mutation failed to show such an arrest. These results are interesting and may be correct but because the authors only used static DNA histograms to

Figure 3. BrdUrd labelling data on the MA13L cells at the start (0 h) of the experiment. In **a**, a histogram of propidium iodide fluorescence corresponding to DNA content is shown. In panel **b**, a dot plot of DNA content against BrdUrd labelling is plotted showing the usual pattern with G₁ and G₂ cells being negative for BrdUrd and all S-phase cells showing positive staining. In panels **c** and **d**, equivalent data are plotted for a sample 6 h after 4 Gy radiation. The DNA histogram **c** shows a reduction of cells in early S-phase whilst cells accumulate in the G₂/M phase as a result of a G₂ block. In **d**, unlabelled cells are shown (small box) leaving G₁ and entering S-phase. In a complete G₁ arrest, this compartment would be empty.

assess cell cycle perturbations at limited time points the results are ambiguous. Their results could be equally well explained by differences, for example, in cell cycle times between the two cell lines. In our study there was a difference of almost 5 h in the potential doubling time of the fastest vs. the slowest cycling cell lines. Goi *et al* (1997) also studied the 282 mutation and compared it with a number of different mutations at codons 286, 133, 132 and 213. They also reported that the 282 mutation retained a G₁-arrest, whilst the cells containing the other mutations did not. Unfortunately, once again, although pulse labelling with BrdUrd was used, analysis was still based only on static methods at a single time point. Thus some reports suggest that heterozygous p53 mutations do not influence the cell cycle related response to radiation (Williams *et al* 1996 and present study), whilst others find changes in some cell lines. The latter findings require confirmation using appropriate dynamic methods. If some LCLs, particularly those with unusual p53 mutations such as those in introns and in the tetramerization domain, do retain p53-mediated cell cycle defects then these lines may have a valuable role in studying the mechanisms by which changes in gene function occur. In our study mutations at codons 175 and 337 did not lead to a change in either cell cycle or apoptotic response in LCLs to radiation. Similarly Williams *et al* (1996) found no defect in G₁-arrest in cell lines with mutations at codons 175, 180, 220 and 248.

The failure to measure defects in cell cycle progression adequately occurs in many other studies unrelated to EBV-transformed lymphocytes. The vast majority of molecular biology studies, in which the role of particular cell cycle regulatory genes is investigated, use only static methods to assess cell cycle progression delays. This can lead, as with the studies on LCLs, to confusion and potential misinterpretation of results. Thus the need to use appropriate methods to measure cell cycle progression is widespread and important.

ACKNOWLEDGEMENT

Lymphoblastoid cell lines as described in Table 1 were kindly donated by John Boyle at the Christie Hospital in Manchester and Nigel Spurr, then at ICRF Clare Hall Laboratories. This work was supported in part by grants from the Medical Research Council and the Association for International Cancer Research.

REFERENCES

- BEGG AC, McNALLY NJ, SHREIVE DC, KARCHER H (1985) A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* **6**, 620.
- BIRCH JM, HARTLEY AL, TRICKER KJ *et al* (1994) Prevalence and diversity of constitutional mutations in the p53 gene among 21 Li-Fraumeni families. *Cancer Res* **54**, 1298.
- CAMPLEJOHN RS, PERRY P, HODGSON SV *et al* (1995) A possible screening test for inherited p53-related defects based on the apoptotic response of peripheral blood lymphocytes to DNA damage. *Br J Cancer* **72**, 654.
- CLARKE OAR, PURDIE CA, HARRISON DJ *et al* (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* **362**, 849.
- DELIA D, GOI K, MIZUTANI S *et al* (1997) Dissociation between cell cycle arrest and apoptosis can occur in Li-Fraumeni cells heterozygous for p53 gene mutations. *Oncogene* **14**, 2137.
- GILCHRIST R, LOMAX ME, CAMPLEJOHN RS (1998) Cell cycle perturbations and apoptosis in EBV-transformed lymphoblastoid cell lines of differing p53 status following gamma radiation. *Br J Cancer* **78** (Suppl 1), 38.
- GOI K, TAKAGI M, IWATA S *et al* (1997) DNA damage-associated dysregulation of the cell cycle and apoptosis control in cells with germline p53 mutation. *Cancer Res* **57**, 1895.
- LALLE P, MOYRET-LALLE C, WANG Q *et al* (1995) Genomic stability and wild-type p53 function of lymphoblastoid cells with germline p53 mutation. *Oncogene* **10**, 2447.

- LI FP, FRAUMENI JF. (1969) Soft-tissue sarcomas, breast cancer and other neoplasms. *Annals Int. Med.* **71**, 747.
- LITTLE JB, NAGASAWA H, KENG PC, YU Y, LI CY. (1995) Absence of radiation-induced G₁ arrest in two closely related human lymphoblast cell lines that differ in p53 status. *J. Biol. Chem* **270**, 11033.
- LOMAX ME, BARNES DM, GILCHRIST R, PICKSLEY SM, VARLEY JM, CAMPLEJOHN RS. (1997) Two functional assays employed to detect an unusual mutation in the oligomerisation domain of p53 in a Li-Fraumeni like family. *Oncogene* **14**, 1869.
- LOMAX ME, BARNES DM, HUPP TR, PICKSLEY SM, CAMPLEJOHN RS. (1998) Characterisation of p53 oligomerization domain mutations isolated from Li-Fraumeni and Li-Fraumeni like family members. *Oncogene* **17**, 643.
- MALKIN D, LI FP, STRONG LC *et al.* (1990) Germline p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* **250**, 1233.
- OKAN I, WANG Y, CHEN F *et al.* (1995) The EBV encoded LMP1 protein inhibits p53-triggered apoptosis but not growth arrest. *Oncogene* **11**, 1027.
- QUINN CM, WRIGHT NA. (1990) The clinical assessment of proliferation and growth in human tumours: evaluation of methods and applications as prognostic variables. *J. Pathol* **160**, 93.
- VARLEY JM, MCGOWAN G, THORNCROFT M *et al.* (1997) Germ-line mutations of *TP53* in Li-Fraumeni families: an extended study of 39 families. *Cancer Res.* **57**, 3245.
- WILLIAMS KJ, HEIGHWAY J, BIRCH JM, NORTON JD, SCOTT D. (1996) No defect in G₁/S cell cycle arrest in irradiated Li-Fraumeni lymphoblastoid cell lines. *Br. J. Cancer* **74**, 698.