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Radiation-induced G_1 arrest is not defective in fibroblasts from Li-Fraumeni families without *TP53* mutations

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Summary Radiation-induced G_1 arrest was studied in four classes of early passage skin fibroblasts comprising 12 normals, 12 heterozygous (mut/wt) *TP53* mutation-carriers, two homozygous (mut/–) *TP53* mutation-carriers and 16 strains from nine Li-Fraumeni syndrome or Li-Fraumeni-like families in which no *TP53* mutation has been found, despite sequencing of all exons, exon–intron boundaries, 3′ and 5′ untranslated regions and promoter regions. In an assay of p53 allelic expression in yeast, cDNAs from these non-mutation strains behaved as wild-type p53. Using two different assays, we found G_1 arrest was reduced in heterozygous strains with mis-sense mutations and one truncation mutation, when compared to the range established for the normal cells. Heterozygous strains with mutations at splice sites behaved like normal cells, whilst homozygous (mut/–) strains showed either extremely reduced, or no, arrest. Strains from all nine non-mutation families gave responses within the normal range. Exceptions to the previously reported inverse correlation between G_1 arrest and clonogenic radiation resistance were observed, indicating that these phenotypes are not strictly interdependent.

Keywords: Li-Fraumeni syndrome; G_1 arrest; cell cycle; fibroblasts; radiosensitivity; p53

Classical Li-Fraumeni syndrome (LFS) families have a proband with sarcoma under the age of 45 years, a first-degree relative with any cancer under age 45, and a first-or second-degree relative with either a sarcoma at any age or any other cancer under age 45 years (Li and Fraumeni, 1969; Li et al, 1988). Li-Fraumeni-like (LFL) families conform to a broader definition (Birch et al, 1994) with a proband having any childhood cancer or sarcoma, brain tumour or adrenal cortical tumour diagnosed before age 45 years with one first- or second-degree relative with a typical syndromal cancer at any age, plus a first- or second-degree relative with any cancer under age 60 years. In both syndromes the predominant cancers are bone and soft tissue sarcomas and breast cancer, plus an excess of brain tumours, leukaemia and adrenocortical carcinomas diagnosed under age 45 years. We have reported germline mutations in the tumour suppressor gene, *TP53*, in 14 of 21 LFS families and four of 18 LFL families (Birch et al, 1994; Varley et al, 1997). An understanding of the consequences of the p53 mutations, particularly in the mesenchymal cells, could have implications for the management and counselling of these families.

Pioneering work by Little (1968, 1970) demonstrated that human cells arrest in the G_1 phase of the cell cycle following exposure to ionizing radiation. Subsequently, it was shown that radiation-sensitive fibroblasts from patients with the cancer predisposition syndrome, ataxia-telangiectasia (AT), did not show this response (Little and Nagasawa 1985; Nagasawa et al, 1985). These observations can be explained by the fact that radiation-induced stabilization of p53 expression in AT cells is both delayed and is reduced compared to normal cells, resulting in lower levels of transactivation of the cyclin-dependent protein kinase inhibitor, $p21^{WAF1/CIP1}$, which has been strongly implicated in the permanent arrest of cells in G_1 (Di Leonardo et al, 1994; Dulic et al, 1994).

Recently, we reported that radiation-resistant fibroblasts from Li-Fraumeni (LF) individuals also show reduced G_1 arrest (Williams et al, 1997). We now report an enlarged study of G_1 arrest in which we have compared the variability in responses of 12 fibroblast strains from normal volunteers with that of 30 strains from 20 classic LFS or LFL families. We have used this material to answer the following questions:

1. Permanent G_1 arrest has been reported following irradiation of cells synchronized in G_1 (Little, 1968, 1970) and transient arrest has been reported following irradiation of asynchronous cells (Kastan et al, 1991). Li et al (1995) questioned whether the two conditions gave the same measure of G_1 arrest, but did not answer the question directly. We have directly compared the two methods using the enlarged panel of cells.
2. Do different *TP53* mutations produce different G_1 arrest responses?
3. How does loss of the wild-type (wt) allele affect the response?
4. What is the response in cells from LF families in which no *TP53* mutation has been found despite exhaustive sequencing of all exons, exon–intron boundaries, 3′ and 5′ untranslated regions and the promoter region (Varley et al, 1997)?
5. To what extent is reduced G_1 arrest a predictor of clonogenic radioresistance?

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Table 1 Donor details, allelic expression and the magnitude of radiation-induced G_1 arrest in fibroblast cultures

Strains	[a]Family (person)	Type	Mutation	Age at biopsy	Sex	[b]FASAY	[d]Transient arrest		[e]Permanent arrest	
							[c]*n*	0.5 Gy	[c]*n*	4 Gy
Group 1 – Normal controls										
83MA				34	F		1	62.6	90.9	2	85.1 ± 4.0
85MA				28	M	4 (wt/wt)	1	66.6	92.5	2	50.2 ± 0.8
93MA				19	M	4 (wt/wt)	1	61.5	89.7	1	67.4
105MA				58	M		1	67.8	95.7	1	74.1
120MA				47	F		1	50.0	86.4	2	74.7 ± 7.4
136MA	83 (I-1)	[f]Prior to		77	M		1	54.2	81.4	1	62.1
141MA	83 (I-2)	mutation		77	F		1	–	85.7	1	76.6
156MA				35	F		6	57.1 ± 3.9	91.0 ± 6.0	1	66.1
162MA				27	F		1	53.8	90.3	1	69.5
176MA				31	F		8	55.4 ± 10.3	94.3 ± 1.8	2	71.8 ± 1.9
177MA	2635 (III-1)	[g]Spouse		68	M	4;4 (wt/wt)	1	72.6	97.1	2	80.3 ± 2.8
187MA	86	[g]Spouse		42	M		1	59.4	93.0	1	90.9
Group 2 – Mutation-carrier families (mut/wt strains)										
FH1	266 (II-4)	LFS aff	R248W/wt	20	M		1	12.5	54.6	3	29.2 ± 7.1
163MA	266 (II-2)	LFS aff	R248W/wt	31	F		1	10.2	59.3	1	27.5
131MA	222 (IV-1)	LFS aff	R248Q/wt	18	M		1	24.5	25.5	1	25.2
138MA	83 (III-4)	LFS unaff	R175H/wt	16	M	45 (mut/wt)	1	28.1	73.5	1	39.2
124MA	16 (IV-1)	LFS aff	Y220C/wt	13	F	65 (mut/wt)	1	12.5	45.8	3	14.9 ± 7.0
109MA	85 (III-7)	LFL unaff	E180K/wt	52	M	49 (mut/wt)	1	35.6	84.2	2	57.1 ± 4.6
110MA	85 (IV-1)	LFL aff	E180K/wt	18	F	58 (mut/wt)	2	43.0 ± 1.6	84.7 ± 5.2	3	51.3 ± 10.7
168MA	1502 (III-1)	LFL aff	R273H/wt	31	F		1	28.7	69.1	–	
178MA	2635 (IV-8)	LFL aff	spl.donor exon 4/wt	39	F	3 (wt/wt)	1	67.5	93.5	4	63.5 ± 10.7
191MA	86 (V-5)	LFS aff	spl. acc. intron 3/wt	16	M		1	52.1	88.2	–	
193MA	2612 (III-1)	LFS aff	R209stop/wt	41	M	12,12 (wt/wt)	1	43.8	75.2	1	44.8
194MA	64 (III-5)	LFS unaff	P152L/wt	42	F	57,56 (mut/wt)	1	3.0	59.8	1	54.6
Group 3 – Mutation-carrier families (mut/–strains)										
161MA-F	7379 (III-6)	LFS aff	L344P/-	45	M	97 (mut/–)	1	–7.1 ± 12.8	–3.7 ± 7.8	1	14.1
172MA	i	LFS aff	R337C/-	34	F		1	–17.7	–1.9	1	19.5
Group 4 – Non-mutation carriers										
140MA	22 (III-4)	LFS aff		14	F	5 (wt/wt)	1	60.5	72.2	3	78.6 ± 11.3
146MA	80 (IV-19)	LFL aff		65	F	4 (wt/wt)	1	47.8	61.2	1	67.3
147MA	80 (V-4)	LFL aff		42	F	4 (wt/wt)	1	76.1	92.5	4	72.8 ± 10.1
148MA	80 (V-5)	LFL unaff		38	M		1	62.4	94.1	1	88.4
154MA	80 (V-6)	LFL aff		36	F		1	62.0	96.1	1	56.1
79MA	81 (III-5)	LFS aff		70	F	9 (wt/wt)	1	70.0	96.5	2	72.5 ± 0.5
80MA	81 (IV-1)	LFS aff		45	M	12;9;4 (wt/wt)	1	64.6	94.6	1	58.7
81MA	81 (IV-3)	LFS unaff		40	F	9 (wt/wt)	1	47.6	90.6	3	56.4 ± 2.1
115MA	82 (IV-5)	LFS aff		21	M		–	–	–	1	70.0
126MA	88 (II-2)	LFS aff		29	M	5 (wt/wt)	2	69.1 ± 9.8	93.8 ± 0.4	2	74.4 ± 0.5
130MA	88 (I-1)	LFS unaff		59	M		1	28.0	71.0	2	85.1 ± 8.0
121MA	119 (II-2)	LFS aff		43	M	8 (wt/wt)	1	45.6	75.0	1	75.2
122MA	119 (II-1)	LFS aff		46	M		1	47.4	76.3	1	73.2
127MA	338 (III-2)	LFL aff		38	F	7;7 (wt/wt)	1	58.8	92.6	2	78.9 ± 4.9
128MA	348 (III-2)	LFL unaff		43	F	26;15;8(wt/wt)	1	65.5	95.7	1	79.4
107MA	353 (III-3)	LFL aff		34	F	6 (wt/wt)	1	45.2	90.3	1	77.5

[a]Family and person numbers as listed in Varley et al (1997); [b]% red (mutant) colonies obtained from separate cDNA preparations with the inferred allelic expression in parentheses; [c]*n*, number of determinations; [d]Values are % inhibition of S phase; [e]Values are % reduction of labelled cells; [f]Parents of de novo mutation; [g]Uninvolved spouse; [h]Cancer affected (aff) or unaffected (unaff) at time of biopsy; [i]Barnes et al (1992). M, male; F, female.

MATERIALS AND METHODS

Fibroblast strains and clonogenic survival

Most fibroblast strains used here and their culture conditions have been described in detail (Boyle et al, 1998; Varley et al, 1998). The origins of additional strains are given in Table 1. Early passage cultures were used in the experiments reported. Clonogenic survival following exposure to low-dose rate (0.011 Gy per min) ^{60}Co radiation was measured as previously described (Boyle et al, 1998).

Measurement of G_1 arrest

Radiation-induced G_1 arrest was determined by two methods. Transient arrest was determined essentially as described by Kuerbitz et al (1992). Confluent cultures were subcultured by 1:3 dilution approximately 3 days before trypsinization. Asynchronous cell suspensions at 1×10^6 per ml were irradiated at room temperature with 0, 0.5 or 4 Gy ^{137}Cs gamma radiation at a dose rate of 3.3 Gy per min, then 1 ml aliquots were inoculated into T150 flasks (Falcon) and incubated at 37°C for 17 h. BrdU was added to 10 μM

and incubation was continued for 4 h, when the cells were harvested by trypsinization, washed in phosphate buffered saline (PBS), resuspended and fixed in 70% ethanol. Fixed suspensions were stored at –20°C prior to staining for FACS analysis. Suspensions were treated at room temperature with 2 N hydrochloric acid for 30 min, then neutralized with borate buffer. Cells were stained by sequential adsorption of mouse anti-BrdU monoclonal antibody, clone BU20a, and rabbit anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC; both from Dako A/S, Denmark). Incorporation of BrdU was analysed by FACS on a FACSCAN using Cellquest (Becton-Dickinson) software.

Permanent G_1 arrest was determined according to Williams et al (1997). Monolayer cultures were held confluent for 10–14 days prior to 48 h incubation in medium containing 0.1% fetal calf serum. The G_1 synchronized monolayers were irradiated with 0 or 4 Gy ^{137}Cs gamma radiation (3.3 Gy per min), trypsinized and plated at 4×10^4 cells per 3 cm diameter petri dish with 1 μCi [^{3}H]-TdR (20 Ci per mM; New England Nuclear). Five plates at each dose were fixed at intervals between 60 and 120 h post-irradiation incubation, the period corresponding to the maximum cumulative labelling index. Plates were coated with Ilford K5 photographic emulsion and stored at 4°C for about 2 weeks prior to developing and staining with Giemsa. The mean fraction of cells with labelled nuclei was determined for each set of five plates and the percentage of inhibition of S phase was calculated by comparing the labelled fractions of the irradiated and unirradiated cells.

Functional assay of alleles in yeast (FASAY)

Messenger RNA was extracted from early passage cells and cDNA was derived and used to test the ability of p53 to transactivate a target gene as previously described (Flaman et al, 1995; Lomax et al, 1997).

Statistical analysis

Statistical analysis was performed using the SPSS statistics package. Fibroblast strains were grouped as normal, LF with mutation and LF without mutation, according to their origin and *TP53* status. G_1 arrest was analysed using the Mann–Whitney *U*-test to

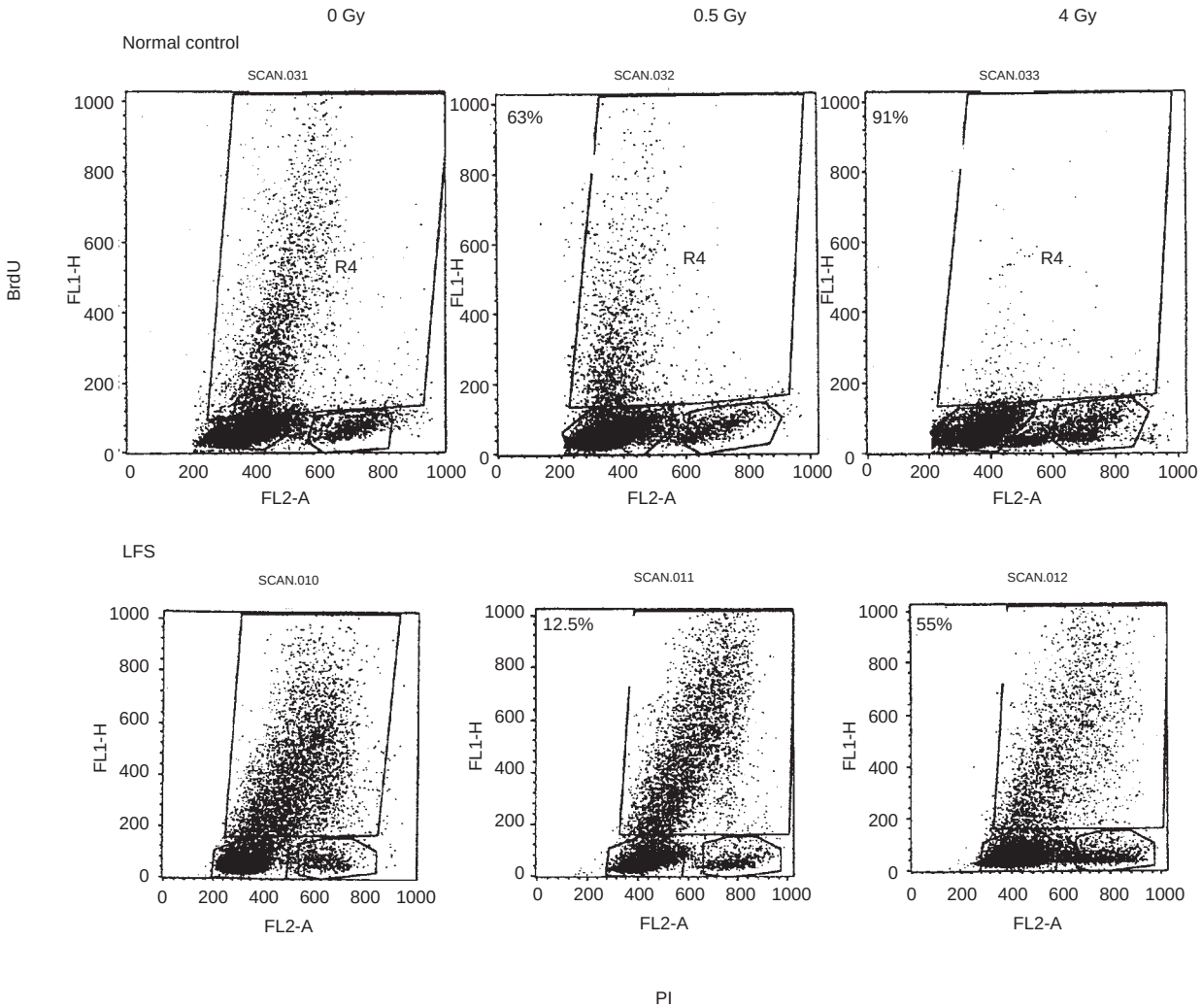

Figure 1 FACS profiles of BrdU labelled cells. Cells were irradiated as indicated and labelled with BrdU under conditions of transient arrest (Materials and methods). Panels show the bivariate distributions and gating of unlabelled G1 and G2 cells and the cells that were in S phase at the time of labelling. Values show the percentage inhibition of S phase in a normal control (83MA) and an LFS strain (FH1)

compare the group means of the aggregated data of all assays. Transient and permanent arrest data were compared using Spearman's rank correlation.

RESULTS

Experiments were performed on four groups of cells represented by 12 strains derived from normal individuals, 12 *TP53* heterozygous strains from LFS and LFL families, two strains with *TP53* mutations that had lost the wild-type allele and 16 strains derived from nine non-mutation families (Table 1).

Allelic expression in yeast

As an additional test for the absence of *TP53* mutations in cells derived from families in which no mutations had been detected by sequencing, fibroblast-derived cDNA was used to test the ability of p53 to transactivate a target gene in yeast cells. Fibroblasts from classic LFS families having mis-sense mutations in the DNA binding region of p53 were used as positive controls (e.g. codons 180, 220) and resulted in an approximately 1:1 ratio of mutant:wt colonies (49–65% mutant; Table 1). Strain 161MA-F, which showed loss of the wild-type allele (mut/–) gave virtually 100% red (mutant) colonies. Cells from normal individuals acted as negative controls and yielded a background frequency of red colonies of < 10%. A mutation affecting splicing (178MA), and a mutation in codon 209 converting it to a stop codon (193MA), were not detected as mutant in the transactivation assay. We used the assay to test p53 expression in fibroblasts from each of the nine LF families in which no *TP53* mutations had been found. In no case was a 1:1 ratio of colonies observed and seven families gave < 10% red colonies indicative of wt p53. However, cells from two families, represented by 80MA and 128MA, consistently gave somewhat elevated frequencies of red colonies, but not approaching a 1:1 ratio. Also, when compared with our experience of normal cells or mis-sense LFS, there was greater variability in the range of values obtained with different cDNA preparations of these cases.

G_1 checkpoint

The effect of mutations in the p53 gene on the G_1 checkpoint was determined by comparing the proportion of cells in S phase following ^{137}Cs radiation. Two independent sets of data were acquired: transient arrest in asynchronous cells was determined by FACS analysis of BrdU incorporation, permanent arrest in cells synchronized in G_1 at the time of irradiation was determined by autoradiography of [^{3}H]TdR uptake.

Transient arrest

Figure 1 shows examples of the DNA profiles and gating of the FACS analyses of a normal control (83MA), and LFS strain FH1 (R248W/wt). The percentage reduction of BrdU-labelled cells occurring as a result of prior exposure to 0.5 and 4 Gy radiation are listed for all strains tested in Table 1. Multiple repeats were performed on two normal strains (156MA and 176MA), otherwise single determinations were performed on most strains.

Permanent arrest

Table 1 also shows the radiation-induced reduction of labelled cells due to permanent arrest in the G_1 phase. The data shown are a combination of those previously reported (Williams et al, 1997) and new data acquired under the same conditions in this study.

Statistical analysis of G_1 arrest data

Comparison of transient and permanent arrest data obtained for all strains after 4 Gy showed a significant correlation (Spearman's correlation coefficient = 0.768, significant at the 0.01 level). However, the significance was principally due to the strong correlation correlation (Spearman's correlation coefficient = 0.879,

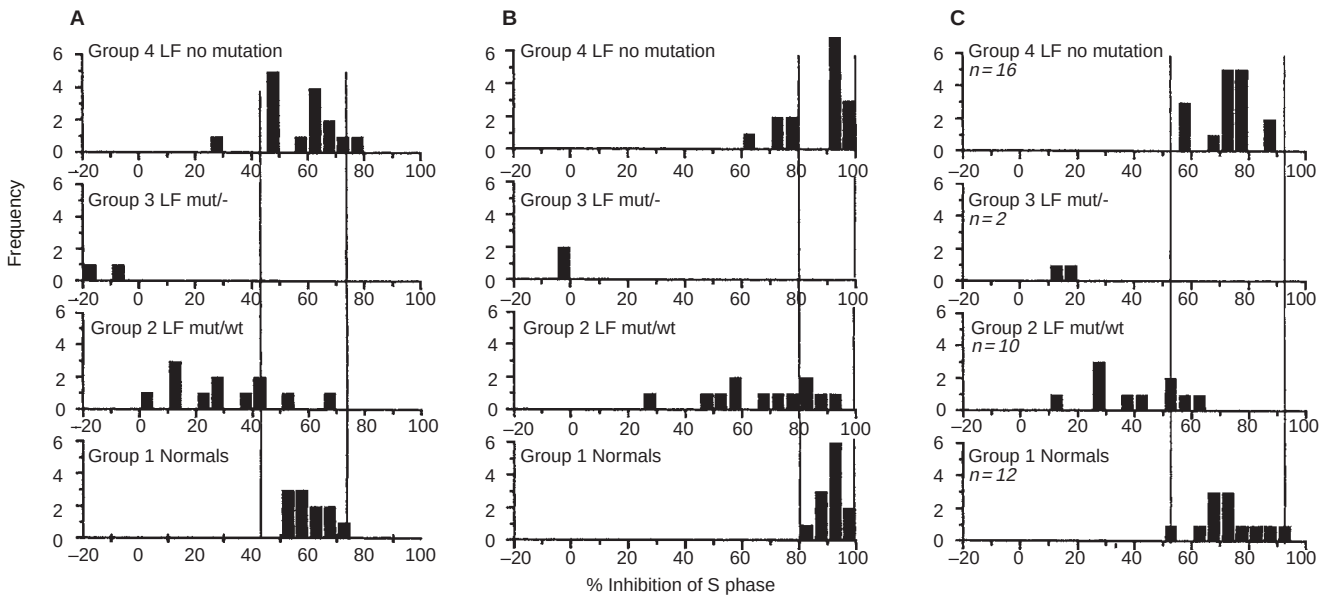

Figure 2 Histograms of inhibition of S phase under conditions of transient arrest after 0.5 Gy (**A**) and 4 Gy (**B**) or permanent arrest after 4 Gy (**C**). Cells are grouped according to *TP53* status. Mean values from Table 1 were binned at 5% intervals and vertical lines indicate 2 standard deviation limits of the mean values of normal control group 1

Table 2 Analysis of G_1 arrest data

Summary of statistics

Group	Transient arrest						Permanent arrest		
	0.5 Gy			4 Gy			4 Gy		
	[a]*n*	[b]Mean	SD	*n*	Mean	SD	*n*	Mean	SD
1. Normals	11	60.09	6.88	12	90.67	4.46	12	72.25	10.61
2. LF, wt/mut	12	30.13	19.20	12	67.79	19.78	10	40.74	16.07
3. LF mut/–	2	–12.40	7.49	2	–2.80	1.27	2	16.80	3.82
4. LF, no mutation	15	46.21	22.97	15	77.76	24.31	16	72.79	9.41

***P*-values of Mann–Whitney *U*-tests**

Groups compared	Transient arrest		Permanent arrest
	0.5 Gy	4 Gy	4 Gy
1 v 2	0.001	0.001	< 0.001
1 v 4	0.59	0.83	0.69
2 v 4	0.001	0.005	< 0.001

[a]Number of strains in group; [b]Mean of aggregated means of percentage inhibition of S phase.

significant at the 0.001 level) found for the mut/wt strains (Group 2, Table 1). These data were linearly correlated over a wide range from approximately 20% to 90%. No significant correlations were found for groups 1 and 4 (normals and non-mutants) whose data points lay mainly at the top end of the linear response, clustered between approximately 60–90%.

Variability in the results obtained with the four groups of cells tested is shown graphically in Figure 2. Mann–Whitney *U*-tests were used to compare the group means (Table 2). G_1 arrest, both transient and permanent, was significantly reduced in heterozygous LF cells when compared to both normal and non-mutation LF cells. In contrast, there was no significant difference between normal and non-mutant LF cells in either of the assays.

Correlations between G_1 arrest and clonogenic radiosensitivity

The relative clonogenic survivals following exposure to 3 and 6 Gy of low-dose rate ^{60}Co radiation were 34.4 ± 7.1 and 19.5 ± 10.4 for 193MA, and 45.3 ± 16.5 and 17.9 ± 4.5 for 194MA (mean percentage survivals ± 1 standard deviation of three independent experiments). Data for the majority of other strains have been reported previously (Boyle et al, 1998; Varley et al, 1998).

From a clinical perspective it may be important to identify abnormal responses for different endpoints in cells from different individuals. As a general strategy, this can be done by assigning as abnormal those responses that fall outside limits set by 2 standard deviations of the mean values of responses obtained from a panel of normal cells. We used this strategy to assess the effects of *TP53* status on G_1 arrest and clonogenic radiosensitivity (Table 3). We defined strains as having compromised G_1 arrest if at least two of the three conditions (transient arrest after 0.5 or 4 Gy and permanent arrest after 4 Gy) showed reduced arrest. We defined as radioresistant any strain whose clonogenic survival was greater than the normal limits (41.8% or 14.0%) after exposure to either 3 Gy or 6 Gy, respectively (Boyle et al, 1998).

The effect of *TP53* status on the relationship between G_1 arrest and clonogenic radiosensitivity, is shown in 2 × 2 concordance tables (Table 4), from which it is apparent that there are several discordant strains in each LF group. Thus both mut/– strains (161MA-F, 172MA) have reduced G_1 arrest but normal radiosensitivity. The dissociation of reduced G_1 arrest and radioresistance was also seen in some heterozygous strains and some strains without p53 mutations.

Among heterozygous strains, there were two discordant strains, 110MA (E180K/wt) which had normal G_1 arrest but was radioresistant, and 131MA (R248Q/wt) which had reduced G_1 arrest and normal radiosensitivity. However, two of ten mutation carrying strains behaved like normal cells in their response to ionizing radiation, having normal G_1 arrest and radiosensitivity, and six had reduced G_1 arrest and were radioresistant, thus conforming to our original concordance (Table 4).

Of six non-mutation strains tested for both parameters, three (80MA, 81MA, 126MA) were discordant and had normal G_1 responses associated with borderline survival responses (Table 3). The three concordant strains, 146MA, 154MA and 79MA, had normal G_1 and survival responses. Within family 81, all three strains tested had normal G_1 response, but 80MA and 81MA were slightly more resistant than 79MA and were differently categorized.

DISCUSSION

Equivalence of G_1 methodologies

The two methods that we used to determine radiation-induced G_1 arrest in human fibroblast cells showed a highly significant correlation for data from heterozygous LF cells with *TP53* mutations (mut/wt) cells, for which there was a linear response over a wide range of values after 4 Gy exposure. However, permanent G_1 arrest appears to be more reliable than transient arrest as a means of discriminating between mut/wt cells and those from families with no mutation (Table 3). Exposure to doses less than 4 Gy may improve the correlation between the two methods for normal and non-mutation groups by inducing less than saturating responses in these cells.

Table 3 Comparison of G_1 arrest and clonogenic radiosensitivity in LF cells compared to normals

Strain (family)	[a]G_1 Arrest compared to normal controls			
***TP53* status**	**Transient**		**Permanent**	[b]**Cell survival following 3 Gy/6 Gy**
	0.5 Gy	**4 Gy**	**4 Gy**	
Group 2 – Mutation carriers				
FH1 (266) R248W/+	r	r	r	R/R
163MA (266) R248W/+	r	r	r	R/R
131MA (222) R248Q/+	r	r	r	n/n
138MA (83) R175H/+	r	r	r	R/R
124MA (16) Y220C/+	r	r	r	R/R
109MA (85) E180K/+	r	n	n	n/n
110MA (85) E180K/+	r	n	n (0.3)	R/R
168MA (1502) R273H/+	r	r	nd	nd
178MA (2635) spl. Don. Exon 3/+	n	n	n	n/n
191MA (86) spl. Acc. Intron 3/-	n	n	nd	nd
193MA (2612) R209stop/+	r	r	r	n/R
194MA (64) P152L/+	r	r	n	R/R
Group 3 – Mut/- strains				
161MA-F (7379) L344P/-	no arrest	no arrest	r	n/n
172MA R337/-	no arrest	no arrest	r	n/n
Group 4 – Non-mutation families				
140MA (22)	n	r	n	nd
146MA (80)	n (1.6)	r	n	n/n
147MA (80)	n	n	n	nd
148MA (80)	n	n	n	nd
154MA (80)	n	n	n	n/n
79MA (81)	n	n	n	n/n
80MA (81)	n	n	n	R/n
81MA (81)	n (1.4)	n	n	R/n
115MA (82)	nd	nd	n	nd
126MA (88)	n	n	n	n/R
130MA (88)	r	r	n	nd
121MA (119)	n (0.6)	r	n	nd
122MA (119)	n (1.2)	r	n	nd
127MA (338)	n	n	n	nd
128MA (348)	n	n	n	nd
107MA (353)	r (1.0)	n	n	nd

[a]n, within normal range; r, reduced response, values in parenthesis show the percentage by which marginal cases (within 2% of the lower limit of the normal range) fall within the given class. [b]n, within normal range; R, resistant; nd, not determined.

Effect of *TP53* mutations on G_1 arrest

Among the mutation-carrying strains, those with mis-sense mutations in the DNA binding region of p53 all showed reduced G_1 arrest, although this was marginal for two strains of family 85 (109MA, 110MA) with a mutation in codon 180. Two strains heterozygous for splice site mutations (178MA, 191MA) gave responses within the normal range. This was probably not due simply to reduced gene dosage as a consequence of truncation of the product of the mutated alleles, because the stop codon mutation in strain 193MA resulted in a reduced response.

Loss of the wt p53 allele drastically reduces G_1 arrest

Two strains (161MA-F, 172MA) that had lost the wt p53 allele (mut/–) but retained mutations in codons 344 and 337, respectively, showed no arrest in the transient assay and greatly reduced arrest when measured at late times in the permanent assay. This confirms a similar finding in MDAH087, homozygous for a mutation in codon 248 (Dulic et al, 1994; Tainsky et al, 1995). Mutations in codons 344 and 337 affect the oligomerization of p53 (Davison et al, 1998). In vitro L344P prevents dimerization and binding to the p53 DNA consensus sequence, whereas R337C has

Table 4 Concordance between G_1 arrest and clonogenic radiosensitivity

Group	Number of strains	G_1 arrest	Radiation survival	
			Normal	Resistant
LF mut/wt	10	Normal	2	1
		Reduced	1	6
LF mut/-	2	Normal	0	0
		Reduced	2	0
LF non-mutation	6	Normal	3	3
		Reduced	0	0

a lowered thermal stability such that tetramer formation and hetero-oligomerization with wt p53 are greatly impaired at physiological temperatures. Since tetramerization is required for transactivation of p53 target genes, the reduced G_1 arrest seen in 161MA-F and 172MA is consistent with a failure to induce $p21^{WAF1/CIP1}$.

G_1 arrest is not defective in LF families without *TP53* mutations

A major observation of this study was the finding that strains from all nine families without *TP53* mutations (Group 4, Table 3) showed normal checkpoint responses in at least two, and usually all, of the three conditions assayed. Direct sequencing of DNA from normal tissue from donors of these cells had failed to detect any *TP53* mutations (Varley et al, 1997) but it was possible that the fibroblast cultures derived from these donors might have acquired mutations in vitro, as had been observed previously (Mirzayans et al, 1995). To check this possibility we tested cells at passage numbers comparable to those used in the G_1 arrest experiments using the FASAY (Table 1) which permits detection of expressed wt and mis-sense mutant p53 alleles (Flaman et al, 1995; Lomax et al, 1997). As expected, germline mis-sense mutations yielded about 50% mutant colonies in the yeast functional assay, whereas the splice site mutation in 178MA yielded 3% mutant colonies, which was within the background frequency of ≤ 5% obtained with wild-type normal cells. Most of the strains from families without *TP53* mutations gave mutant colonies at frequencies within, or close to, the normal range. However, 80MA and 128MA gave increased numbers of mutant colonies with some cDNA preparations but not others, the reason for which is unknown. The increased number of mutant colonies did not appear to be associated with reduced G_1 arrest since both strains showed normal G_1 arrest.

G_1 arrest is not a consistent predictor of clonogenic radiation resistance in LF fibroblasts

In a previous study (Williams et al, 1997) we demonstrated an inverse correlation between the extent of G_1 arrest and radiosensitivity measured after exposure to ionizing radiation given at either high- or low-dose rates. Because exposure to low-dose rate radiation provides greater discrimination of sensitive and resistant cell populations in the clonogenic assay, we used this method to expand the number of strains studied. Our original results contrasted cells from normal individuals which had, by definition, normal G_1 arrest and radiosensitivity, with Li-Fraumeni cells mainly from mutation carriers, which had reduced G_1 arrest and were radioresistant. However, in strains that had lost the wild-type allele (172MA, R337C/- and 161MA-F, L344P/-) there was reduced G_1 arrest but normal radiosensitivity, clearly showing that the two events are dissociated in the absence of intact p53 (Table 3). The lack of G_1 arrest in p53 mut/– strains mimics that of AT homozygotes (Nagasawa et al, 1985), which are deficient in p53 stabilization following irradiation (Kastan et al, 1992). A similar effect is seen in embryo (Westphal et al, 1997) and adult skin (JMB and MJG, unpublished observations) fibroblasts from ATM and p53 knockout mice. However, AT homozygotes are more sensitive to ionizing radiation whereas p53 mut/– cells have normal radiosensitivity (Boyle et al, 1998).

A caveat affecting the interpretation of the results is that our ability to make a proper classification is critically dependent on the number of strains used to define normal limits for each of the parameters studied, because the group mean will be less strongly influenced by outliers as the sample size increases. The present sample size (n = 12) is a compromise that allowed a reasonable accuracy with the available material and manpower resources. Nevertheless, these observations do appear to emphasize an apparent variability in phenotypes among Li-Fraumeni cell strains which may be controlled in part by the site of *TP53* mutation and by the genetic background in which it is expressed. Where a mutation produces a strong effect (e.g. R248Q in family 266, Table 3; R175H in family 86, Boyle et al, 1998) cells from different members of the family have similar phenotypes that are sufficiently different from wild-type to place them in different categories. However, where a mutation has a weaker effect, the phenotypes may be close to wild-type and small differences in genetic background may place different individuals into different categories. This is illustrated by family 85 (E180K) where strains 109MA and 110MA both showed normal G_1 arrest, but 110MA was slightly more resistant than 109MA and appeared discordant in comparison.

Cellular phenotypes and p53 involvement in non-mutation families

The nine families without *TP53* mutations included both classical LFS (five families) and LFL (four families) patterns of disease. Within these families the most likely individuals to be carrying a genetic predisposition towards cancer are those affected by cancer. The majority of strains (12 of 16) used were from affected individuals, so it is highly probable that we are mainly looking at strains carrying genetic predisposition towards cancer. Our results provide no clear evidence to suggest that defective p53 expression is the cause of cancer predisposition in these families.

Recently, a number of genes have been identified that have homology with p53, e.g. p73, p53CP and KET (Bian and Sun, 1997; Jost et al, 1997; Kaghad et al, 1997; Schmale and Bamberger, 1997). Superficially, p73 is an attractive candidate since, like p53, it is a sequence-specific transactivator that probably requires oligomerization, and overexpression of p73 induces p21 and blocks cell proliferation (Kaghad et al, 1997). But, unlike p53, p73 is neither stabilized nor activated by DNA damage although in other ways it fulfills many of the functions of p53 (Oren, 1997). Expression of p73 and KET appear largely restricted to neurological and epithelial tissues, respectively. Thus, at present, there may be a possibility that cancer predisposition in p53 non-mutation families could result from mutations in these genes, or in other homologues still to be discovered, in which case

cells from these families might be expected to show normal G_1 arrest due to an intact p53 response to DNA damage, such as we have observed in these families.

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