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Brief Communication

Immortalization of Four New Fanconi Anemia Fibroblast Cell Lines by an Improved Procedure

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Abstract—*Fanconi anemia (FA) is an autosomal recessive disease characterized by birth defects, progressive bone marrow failure and increased risk for leukemia. FA cells display chromosome breakage and increased cell killing in response to DNA crosslinking agents. At least 5 genes have been defined by cell complementation studies, but only one of these, FAC has been cloned to date. Efforts to map and isolate new FA genes by functional complementation have been hampered by the lack of immortalized FA fibroblast cell lines. Here we report the use of a novel immortalization strategy to create 4 new immortalized FA fibroblast lines, including one from the rare complementation group D.*

INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive disease characterized by developmental delay, skeletal abnormalities, progressive bone marrow failure, altered skin pigmentation and increased risk for leukemia and solid tumors (1–3). FA cells have a high frequency of chromosomal aberrations inducible by DNA crosslinking agents such as Mitomycin C (MMC), diepoxybutane (DEB) or cisplatinum. Hence, the basic defect in FA has been proposed to be a defect in repair of DNA-damage by cross-linking agents. FA cells are also hypersensitive to oxygen and have an abnormal cell cycle (4). Somatic cell hybridization experiments have demonstrated the existence of at least five FA complementation groups (5–7). The gene responsible for the defect in FA group C patients was cloned by functional complementation and localized to chromosome 9q22.3 (6, 8). The *fac* gene encodes a protein of 558 amino acid residues with a still unknown function. Mutation analysis of FAC is currently used to identify patients in complementation group C (9–11). The genes responsible for the defects in all other complementation groups have not yet been isolated.

Numerous EBV-transformed lymphoblast and primary fibroblast cell lines from FA patients are available in different cell repositories. However, these types of cell lines are not suitable for functional complementation by microcell mediated chromosome transfer. To date only one SV40 transformed and immortalized FA fibroblast cell line (GM 6914) from a complementation group A patient has been described (5). Additional FA fibroblast cell lines, especially from complementation groups other

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than A and C are required for the isolation of new FA genes and also for the study of their function (12–15).

Here we report the generation of four new immortalized Fanconi anemia fibroblast cell lines by chemical mutagenesis of early passage SV40 transformed fibroblast cells with ethyl methane sulfonate (EMS). These cell lines maintained their FA phenotype and will therefore be useful for functional studies.

MATERIALS AND METHODS

Cells. Primary skin fibroblast cell lines PD20F, PD100F, PD113F, PD134F, PD220F, PD224F, PD238F, PD259F, PD260F and PD261F were established using standard procedures (16). The skin samples were donated by Fanconi anemia patients to the Oregon Health Sciences University (OHSU) Fanconi Cell Repository. Informed Consent Forms were filed from each of these individuals. GM6914 group A cells were purchased from the Institute for Medical Research (Camden, New Jersey). Cells were grown in α-MEM (Gibco) containing 15% fetal bovine serum. Mutational analysis (9, 10) was used to exclude cells from complementation group C.

Primary foreskin fibroblast cells were established from a normal individual and GM639 SV40 immortalized fibroblast cells from a Galactosemia patient were purchased from the Institute for Medical Research (Camden, New Jersey). Both cell lines were used as normal control cell lines.

Immortalization Procedure. Primary human diploid FA fibroblast cultures of early passage number were transfected with pSV7, a defective SV-40 containing plasmid (17–19). 10^7 exponentially growing cells were resuspended in Cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$, pH 7.6) (20) and electroporated with 10 μg of supercoiled plasmid DNA at 960 μF and 300 V in a Biorad gene pulser. Two days after electroporation the amount of serum was reduced from 15% to 10% and the cells were neither trypsinized nor passaged until typical cytological changes characteristic of transformed cells were seen after 30–40 days. After transformation, 10^7 T-antigen positive cells (cells pooled from three to five foci) were mutagenized with 1 μg/ml ethyl methane sulfonate (EMS, Kodak) for 24 hours. Equally dense dishes of untreated transformed and primary fibroblasts served as controls. After mutagenesis cells were fed twice a week and split at or before confluence. All cells were subcultured until they either ceased to grow or achieved immortality which is defined by 100 population doublings (PDL).

Kill Curves. Cell survival following Mitomycin C or diepoxybutane treatment was assayed either by measurement of Colony-forming Efficiency (CFE) or by MTT Assay (MTT: 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Sigma).

For the clonogenic assay, 10^4 cells/100 mm dish were plated and continuously treated with Mitomycin C or diepoxybutane for 4 days. After 10 days of growth without treatment, the cells were fixed and colonies were stained with 0.5% crystal violet (21). For the MTT assay 1000 or 3000 cells were plated in duplicate in 24-well plates. 24 h after seeding Mitomycin C or diepoxybutane were added to the media and the cells were cultured without media change for 4–5 days. Cell viability was assayed colorimetrically (22).

Cell Fusion Experiments. Several clones were picked from each of the four new immortal fibroblast Fanconi cell lines (PD20i, PD220i, PD224i and PD259i). Mitomycin C sensitive clones were electroporated with plasmids containing either the hygromycin (pPGK-hygro) or neomycin (pPo12sneo) resistance markers. Selection was carried out in a final concentration of 150 μg/ml Hygromycin B (Calbiochem) or 200 μg/ml G418 (Sigma) in complete media. Cells were fused by PEG (Sigma) according to standard procedures (23, 24) and selected in complete media containing 400 μg/ml Hygromycin B and 400 μg/ml G418.

Table 1. Chromosome Breakage Results

Cell line	Modal chromosome numbers	% cells with radials ng/ml Mitomycin C			
		0	4	8	40
Parental:					
PD20	42	0	0	0	24
PD220	62	0	46	34	—
PD224	61	0	68	84	—
PD259	59	4	32	50	—
GM6914	62	2	16	40	—
control:					
GM639	104	0	0	0	0
93–2	46	0	0	0	4
Hybrid:					
20–6914	97	0	0	0	4
20–220	98	0	0	0	2
20–224	104	0	0	0	0
20–259	93	0	0	0	0
6914–220	101	2	38	78	—
6914–224	101	0	60	90	—
6914–259	99	2	28	54	—

Chromosomal Analysis

Culture and Harvest. Immortalized fibroblast cell lines were subcultured into T-25 flasks and maintained in their original culture media for 24 hours. Cultures were then exposed to various concentrations of Mitomycin C and diepoxybutane, from 0 to 60 ng/ml and 0 to 200 ng/ml, respectively. Following 48 hours exposure to clastogen, one drop/ml medium of 10 μg/ml colcemid (Sigma) was added to each culture. Harvest of cells began 3 hours later. Cells were spun down at 1000 rpm for 10 min. Cells were resuspended in 0.075 M KCl, spun down again and resuspended in 3:1 = methanol: acetic acid. Fresh fixative was added prior to making slides.

Breakage Analysis. Slides were stained with Wright stain. Initially, at least 50 metaphases were analyzed per cell line per harvest for each of the clastogen concentrations. Optimum concentrations of Mitomycin C and diepoxybutane were established for demonstrating breakage and radial formation for each cell line. For all subsequent studies, only these optimum concentrations were used (i.e. 0, 40 and 60 ng/ml Mitomycin C and 100 and 200 ng/ml diepoxybutane for PD20; 0, 4 and 8 ng/ml Mitomycin C and 10 and 20 ng/ml diepoxybutane for PD220, PD224 and PD259).

Karyotyping. Slides were stained by a trypsin/Wright method (25). At least three karyotypes were prepared per cell line.

RESULTS

Transformation. 3 to 4 weeks after electroporation with pSV7, all cell lines (10/10) showed phenotypic characteristics of transformed cells. At least 3 foci of transformed cells were seen in each flask. In contrast to the original primary culture (initiated with airmix of 0% O_2 and 5% CO_2) all transformed FA fibroblasts became insensitive to oxygen (20% O_2, 5% CO_2). As shown in Table 1 and Fig. 1 all transformed cell lines remained sensitive to Mitomycin C and therefore retained the hallmark of their FA phenotype.

Immortalization. Four out of ten SV40 transformed FA fibroblast cell lines, mutagenized with EMS, showed a permanent growth phenotype and were cultured continuously for over 1 year without any loss in growth rate. They reached more than 150 population doublings (PDL), whereas all controls—primary fibroblast cells and SV40 transformed, not mutagenized cells derived from the same individuals—showed phenotypic characteristics of senescent cells and ceased dividing before they reached 80 population doublings. Thus, 4/10 cell lines with EMS treatment and 0/10 cell lines without chemical mutagenesis escaped crisis and had the characteristics of immortal cells.

Phenotype of the 4 New Cell Lines. The karyotypes of PD20i, PD220i, PD224i and PD259i were abnormal in that the modal chromosome number was aneuploid (Table 1). Chromosome breakage analysis confirmed that the new cell lines displayed increased radial formation in response to clastogenic agents. The degree of sensitivity to Mitomycin C varied between the four cell lines. PD20i was the least sensitive cell line, followed by PD259i, PD224i and PD220i (Table 1).

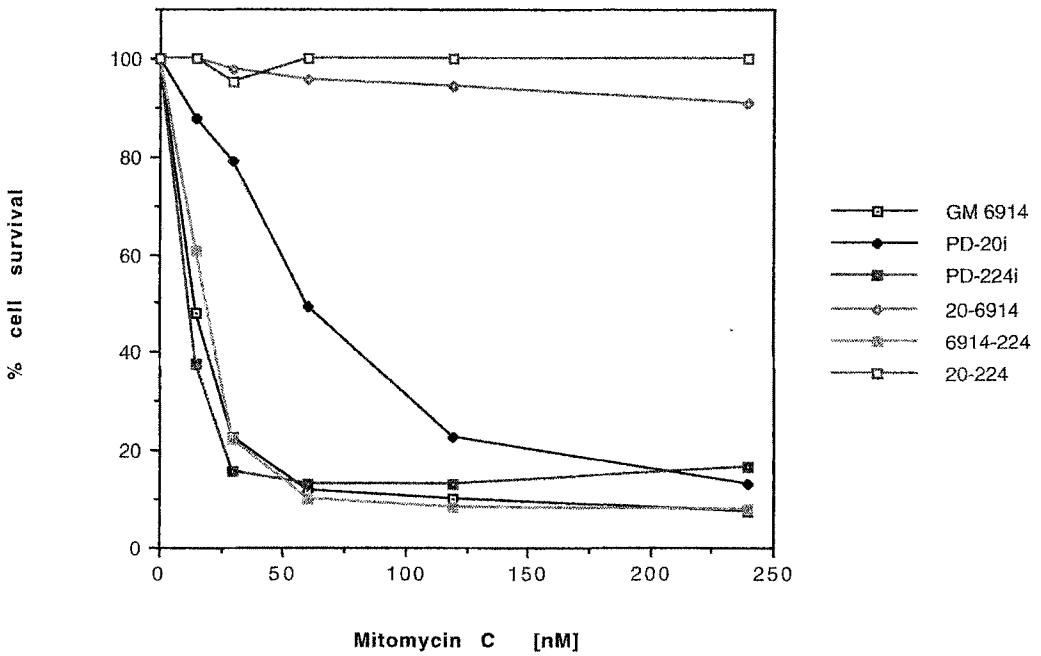

Fig. 1. Mitomycin C kill curves in FA cell hybrids and their immortal parental cell lines.

Complementation Analyses. Table 1 summarizes all cytogenetic results obtained from whole cell fusions between PD20 (Group D) and GM6914 (Group A) and the other 3 immortal FA lines. Figure 1 shows the Mitomycin C and Figure 2 the diepoxybutane concentration response curves of selected parental cell lines and their hybrids.

When the Hygromycin resistant FA(D) cell line (PD20i) was fused with the G418 resistant cell line (GM6914 group A cells), which was derived from an FA(A) patient, the analyzed hybrid was insensitive to Mitomycin C and diepoxybutane. The same results were found after fusion of PD20 with either PD220, PD224 or PD259. Fusion of PD20 with itself (PD20 Hygromycin resistant × PD20 G418 resistant) yielded hybrids that were sensitive to cell killing by DNA crosslinking agents.

The complementation group for patient PD-20 was established, based on fusion of PD-20L lymphoblasts with the group D reference lymphoblast line HSC62NT, as recently described (7). Four independent fusions were carried out, each one resulting in growing hybrid cell populations that were found to be hypersensitive to Mitomycin C in a standard growth inhibition test.

Drug concentrations causing 50% inhibition of growth were 1.2nM (PD-20L) and 8.5nM (HSC62NT) for the parental cell lines, whereas all four hybrid cell populations had IC50 values well below 1nM. Since wild type cell lines typically exhibit values of 20nM or higher, this result implied that the defect was not complemented in this combination of fusion partners. The result was therefore taken as evidence that patient PD-20 belongs to complementation group D.

DISCUSSION

Immortalization. In human fibroblasts spontaneous immortalization is a rare event with a rate of $1/3 \times 10^7$ cell divisions in lung fibroblasts and $1/10^6$ cell divisions in mammary epithelial cells (26–28). Numerous strategies such as UV or X-ray irradiation, infection with various DNA

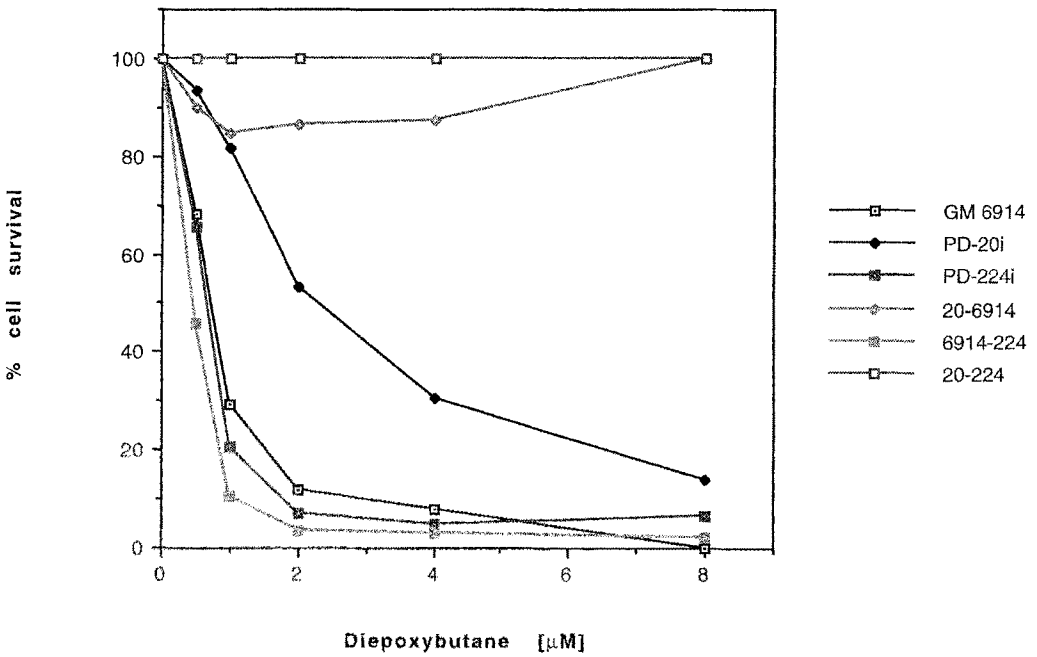

Fig. 2. Diepoxybutane kill curves in FA cell hybrids and their immortal parental cell lines.

tumor viruses or oncogenes and chemical mutagenesis have been used in attempts to improve the low immortalization rate. However, escape from crisis (M2) has been achieved only rarely and a direct relationship between treatment and the immortalization event was not established in the few instances reported (29–32). It has been hypothesized that several independent events are required for immortalization of primary diploid cells (33). Transformation with SV-40 T-antigen alone is insufficient to achieve immortalization, but accomplishes at least one of the required steps. In the past we observed that the frequency of immortalization in SV-40 transformed cells approximates the rate expected from spontaneous mutagenesis (16, 18). We therefore reasoned that an increased mutation rate induced with a chemical agent may increase the likelihood of additional events required for immortalization.

In the study reported here, all Fanconi anemia fibroblast cell lines (10/10) were successfully transformed by pSV7 and their life span could be extended by 20–30 PDL, as has been reported before in the literature (11, 13, 34). Chemical mutagenesis yielded four immortalized fibroblast cell lines emerging from a total of ten SV40 transformed fibroblast cell lines. This success in inactivating or bypassing the mortality stage mechanisms is higher than reported in earlier studies. Our result suggests that transformation of primary fibroblasts with SV40 followed by chemical mutagenesis with EMS indeed may be an effective method for achieving immortalization. At this point we do not know if the DNA repair deficiency and/or chromosomal instability of FA cells is responsible for the success (4/10) presented in this study. Even with the improved method, however, a majority (6/10) of cell lines failed to immortalize. It has been suggested that the aneuploidy induced by T-antigen produces extra copies of a gene, which must be inactivated before immortalization occurs (28). Therefore, the immortalization frequency might be increased if clones from early passage transformed fibroblasts with low modal chromosome number are treated by EMS mutagenesis.

New Fanconi Anemia Cell Lines. The four new FA cell lines generated in this study are useful reagents for functional studies in FA cells as well as for attempts to clone the FA(A) and FA(D) genes. The only immortalized of the FA group D fibroblast cell line reported to date is PD20. All 4 new cell lines retained their sensitivity to growth inhibition by DNA crosslinking agents as well as spontaneous and Mitomycin C-induced chromosome breakage and radial formation. Earlier studies (5) determined that the SV40-transformed FA (A) fibroblast cell line GM6914 group A cells had an ED_{50} of 1 nM Mitomycin C. We found comparably low ED_{50}s of ~5 nM in all 3 new FA (A) cell lines indicating exquisite Mitomycin C sensitivity. In contrast, the ED_{50} of the group D cell line, PD20, was ~10 fold higher at 60 nM Mitomycin C. It is possible that FA complementation group D cells as a group are less sensitive to DNA crosslinking agents than group A cells, but additional group D cell lines will have to be established and studied before this correlation can be made.

Interestingly, whereas primary cells from FA patients exhibit hypersensitivity to oxygen, all transformed and immortalized FA fibroblasts were no longer hypersensitive. This is consistent with earlier findings (4), where the authors predicted the cellular sensitivity to oxygen is a secondary rather than a primary effect of mutations in FA. However, we can not exclude the possibility that the expression of large T-antigen in transformed cells might somehow rescue the cells from oxygen hypersensitivity.

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