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Chromatid damage after G_2 phase x-irradiation of cells from cancer-prone individuals implicates deficiency in DNA repair

(chromatid gaps and breaks)

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ABSTRACT Ten lines of skin fibroblasts from individuals with genetic disorders predisposing to a high risk of cancer were compared with nine lines from normal adult donors with respect to chromatid damage after x-irradiation [25, 50, and 100 rad (0.25, 0.50, and 1 gray)] during G_2 phase. The 10 cell lines represented five genetic disorders: Bloom syndrome, familial polyposis, Fanconi anemia, Gardner syndrome, and xeroderma pigmentosum, complementation groups A(XP-A), C(XP-C), E(XP-E), and variant (XP-Va). The incidence of chromatid breaks in all cancer-prone lines except XP-E and XP-A was significantly higher than in the normal lines. The incidence of chromatid gaps in all cancer-prone lines except XP-A and XP-Va was significantly higher than in the normal lines. Because each chromatid apparently contains a single continuous DNA double strand, chromatid breaks and gaps represent unrepaired DNA strand breaks arising directly or indirectly during excision repair of x-ray-induced DNA damage. These cytogenetic data together with results from use of the DNA repair inhibitor arabinofuranosyl cytosine (cytosine arabinoside) suggest that cells from all of these cancer-prone individuals are deficient in some step of DNA repair, predominantly excision repair operative during the G_2-prophase period of the cell cycle. It appears that these DNA repair deficiencies are associated with a genetic predisposition to a high risk of cancer.

A number of inherited human disorders, including ataxia telangiectasia, Gardner syndrome (GS), familial polyposis (FP), Fanconi anemia (FA), Bloom syndrome (BS), and xeroderma pigmentosum (XP), predispose the individual to a high risk of cancer. Cells from these individuals are useful for elucidating mechanisms of carcinogenesis. A deficiency in the repair of UV-induced DNA damage was described in 1968 (1) in cells from XP individuals with a high incidence of skin cancer. Subsequently, studies on ataxia telangiectasia and FA cells provided some evidence that these cells may be defective in repair of DNA damage produced by ionizing radiation or DNA crosslinking agents (2-9). However, numerous attempts with biochemical methods have failed to reveal a DNA repair deficiency(ies) in all of these genetic disorders. When exposed to ionizing radiation during the G_2 phase of the cell cycle, skin fibroblasts from ataxia telangiectasia and FA individuals show a high incidence of chromatid breaks or gaps at metaphase compared with cells from normal individuals (3, 8, 9). Because each chromatid apparently contains a single continuous DNA double strand, chromatid breaks and gaps represent unrepaired DNA strand breaks (3, 8-17). These strand breaks could arise directly or indirectly during excision repair of the DNA damage produced by ionizing radiation (9, 13, 16). In view of this interpretation of cytogenetic results, cells from at least two of the genetic disorders, ataxia telangiectasia and FA, appear to have a DNA repair defect operative during G_2-prophase.

The present study attempted to quantify chromatid damage after x-irradiation during G_2 phase in skin fibroblasts from donors with BS, FP, FA, GS, or XP (complementation groups A, C, E, and variant) to ascertain whether all of these cancer-prone cells have DNA repair deficiencies that can account for their predisposition to cancer.

METHODS

Cell lines RJH-4, PC-109, BH, and DWsr were generously supplied by R. Trimmer (National Cancer Institute) and line KD was supplied by T. Kakunaga (National Cancer Institute); the other cell lines were obtained from the American Type Culture Collection (Rockville, MD) or the Institute for Medical Research (Camden, NJ). Stock cultures were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum as described (17). Normal cell lines were used at passage levels 6-14; line KD was assayed several times at passages 17-35. The cancer-prone cell lines were used at passage levels 8-15 except GM 0449A and XP 25RO were assayed at passage 20 and GM 2415 was assayed at passage 21.

For experimental studies, 10^5 cells in 2 ml of medium was inoculated into Leighton tubes, each containing a 9 × 50 mm coverslip (no. 1 thickness, Bellco Glass). After 48 hr of incubation at 37°C, cultures were irradiated by using two Philips RT250 opposing therapeutic 250 kV potential x-ray tubes operated at 235 kV, 15 mA, with 0.25-mm Cu and 0.55-mm Al filters (half-value-layer, 0.9 mm Cu); dose rate was 126 R (0.32 C/kg)/min at a target distance of 54 cm. After irradiation, culture fluid was renewed within approximately 10-30 min and Colcemid was added (0.1 μg/ml) for 1 hr or as indicated. The DNA repair inhibitor, arabinofuranosyl cytosine (ara-C; cytosine arabinoside) (Sigma) was added to the cultures at 10 μM immediately after x-irradiation. For chromosome analysis, the irradiated and control cells were processed *in situ* on coverslips by techniques described (18).

Analyses were made on randomized, coded preparations; four cultures were used for each variable, and 100-200 metaphase cells were studied per variable except for three analyses on XP-C (74-86 cells), two analyses on DWsr (76 and 88 cells), and one analysis on PC-109 (58 cells). Abnormalities scored as chromatid breaks showed distinct dislocation and misalignment of the chromatid fragment, whereas gaps or achromatic lesions

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Abbreviations: GS, Gardner syndrome; FP, familial polyposis; FA, Fanconi anemia; BS, Bloom syndrome; XP, xeroderma pigmentosum; ara-C, arabinofuranosyl cytosine.

Table 1. Chromatid damage in normal human skin fibroblasts after x-irradiation at three dosages during G_2 phase

	Gaps, no./cell				Breaks, no./cell			
Cell line	0 rad	25 rad	50 rad	100 rad	0 rad	25 rad	50 rad	100 rad
CRL 1188	0	0.086	0.050	0.085	0.014	0.011	0	0.015
CRL 1191	0	0.048	0.056	0.062	0	0	0.021	0.017
CRL 1221	0	0.082	0.104	0.101	0.007	0.014	0.018	0.019
CRL 1222	0	0.070	0.120	0.131	0.010	0.015	0.005	0.021
CRL 1224	0	0.094	0.094	0.098	0	0.017	0.019	0.045
CRL 1232	0	0.065	0.089	0.087	0.006	0.013	0.025	0.020
RJH-4	0	0.056	0.037	0.080	0.019	0.011	0.019	0.008
KD	0	0.049	0.020	0.047	0	0.008	0.010	0.019
GM 0500	0.005	0.060	0.075	0.065	0.010	0.005	0.005	0.005

showed no dislocation in spite of chromatid discontinuity.

In the statistical analysis of data, χ^2 homogeneity tests were used for comparing cells from the same cell line under different conditions (e.g., to determine if x-irradiation increased the frequency of breaks or gaps in a particular cell line). Comparison of each mutant line to normal was performed by using the *t* test (19). All *P* values reported are two-sided. The *t* tests were based on the average response at the three x-ray doses. For each cell line, the proportion of cells with one or more gaps (or breaks) was computed for each dose. The average of these proportions at 25, 50, and 100 rad was the quantity upon which the statistical analyses reported in the paper were based. Analyses were also performed by using the average of the mean numbers of breaks or gaps per cell at 25, 50, and 100 rad, and these analyses gave similar results (the *P* values tended to be smaller).

Because metaphase cells were examined for chromosome damage within 1.5 hr after x-irradiation, we could be assured that the cells were in G_2 phase at the time of irradiation.

RESULTS

X-Ray-Induced Chromatid Damage in Cells from Normal and Cancer-Prone Individuals. X-irradiation of cells during G_2 produced only two types of chromosome aberrations—chromatid gaps and breaks—apparent at the first post-treatment metaphase. Tables 1 and 2 show the incidence of chromatid gaps and breaks in 9 lines of skin fibroblasts from normal donors and 10 lines from cancer-prone individuals after 0, 25, 50, or 100 rad of x-irradiation during G_2. No consistent or clear-cut dose–response with respect to x-ray-induced chromatid gaps was observed. However, the chromatid damage seen at the first post-irradiation metaphase is the net result of chromosomal DNA damage (which presumably would be dose-related) and the repair of this damage. For those lines susceptible to chromatid breaks, the response was dose-related. The incidence of chromatid breaks in all cancer-prone lines except XP-E and XP-A was significantly higher than in the normal lines: BS, $P < 10^{-4}$; FP, $P = 0.002$; FA, $P = 10^{-4}$; GS, $P = 0.023$ and 0.004; XP-C, $P = .003$; and XP-Va, $P = 0.004$. The incidence of chromatid gaps in all cancer-prone lines with the exception of XP-A and XP-Va was significantly higher than in the normal lines: BS, XP-C, XP-E, FP, and GS (BH), $P \leq 10^{-3}$; FA, $P = 0.002$; GS (DWsr), $P = 0.009$. The cancer-prone lines, excluding XP-A and XP-Va, were homogeneous with respect to x-ray-induced chromatid gaps ($P = 0.100$). The incidence of gaps in XP-Va was higher but not significantly different from that in the normal lines. The two XP-A lines had a significantly lower incidence of x-ray-induced gaps than did the nine normal lines ($P = 0.013$).

To evaluate the influence of time after irradiation on the yield of chromatid aberrations, cells of a normal line (RJH-4) and of XP-E (XP2RO) were exposed to 100 rad and fixed for metaphase analysis at 0.5, 1, 2, 3, and 4 hr after irradiation. No metaphase cells were observed at 2–4 hr, indicating that, like normal cells, the cancer-prone cells manifested a distinct radiation-induced mitotic block between 1 and 2 hr post-irradiation. Furthermore, at 0.5 hr after irradiation the chromatid damage in both lines was similar (Fig. 1). However, at 1 hr after irradiation the incidence of chromatid gaps was decreased in the normal line but increased in the cancer-prone line.

An additional experiment compared cell lines from a normal (RJH-4) and a GS donor (HT 3252) with respect to radiation-induced G_2 mitotic block and rate of progression of G_2 cells into metaphase (Fig. 2). In both cell lines, x-irradiation produced, within 0.5–1 hr, a similar decline in the number of cells en-

Table 2. Chromatid damage in skin fibroblasts from cancer-prone individuals after x-irradiation at three dosages during G_2 phase

		Gaps, no./cell				Breaks, no./cell			
Disorder	Cell line	0 rad	25 rad	50 rad	100 rad	0 rad	25 rad	50 rad	100 rad
BS	GM 2548	0	0.375	0.275	0.325	0.020	0.095	0.140	0.205
FP	PC-109	0	0.416	0.319	0.466	0	0.048	0.043	0.086
FA	GM 0449A	0	0.285	0.340	—	0.015	0.055	0.136	—
GS	BH	0	0.219	0.329	0.441	0	0.026	0.030	0.051
	DWsr	0	0.155	0.148	0.288	0	0.019	0.034	0.098
XP-C	CLR 1158 XP 8BE	0.012	0.257	0.431	0.460	0	0.014	0.059	0.081
XP-E	GM 2415 XP 2RO	0.010	0.221	0.332	0.403	0.015	0.016	0.011	0.007
XP-Va	CRL 1162 XP 4BE	0	0.126	0.112	0.108	0.007	0.042	0.033	0.072
XP-A	CRL 1223 XP 12BE	0	0.033	0.041	0.035	0.015	0.011	0.012	0.015
XP-A	CRL 1261 XP 25RO	0.005	0.023	0.020	0.006	0.010	0.012	0.010	0.017

tering metaphase followed by a G_2 mitotic block between 1 and 1.5 hr post-irradiation.

Influence of ara-C on X-Ray-Induced Chromatid Damage. ara-C, an inhibitor of the repair polymerase, was added to normal cells and XP-A cells to ascertain whether the low incidence of radiation-induced chromatid gaps in XP-A cells resulted from proficient repair, as in normal cells, or from deficient excision repair; XP-A cells are known to be deficient in endonucleolytic incision of UV-damaged DNA. In the normal cell line, exposure to ara-C after x-irradiation in G_2 significantly increased the incidence of chromatid gaps and breaks ($P = 10^{-4}$ and 0.009, respectively), whereas in XP-A cells no such increase was observed (Table 3). From these observations, we conclude that the low incidence of chromatid gaps in XP-A cells results from deficient endonucleolytic incision of DNA, an initial step in nucleotide excision repair.

DISCUSSION

A major finding of this study is that lines of skin fibroblasts from individuals with genetic disorders associated with a high risk of cancer show a higher incidence of chromatid damage after x-irradiation during G_2 phase than do cells from normal adult donors. Cells of all lines were predominantly diploid and were of comparable density at the time of x-irradiation. Of five disorders, BS, FP, FA, GS, and XP (three complementation groups and the variant), all but XP-A and XP-Va cells exhibited a significantly higher frequency of chromatid gaps than normal cells when irradiated in G_2 or within 1.5 hr of metaphase. The incidence of x-ray-induced chromatid breaks in all cancer-prone lines except XP-A and XP-E was also significantly higher than in the normal cells. Additionally, cells from another cancer-prone genetic disorder, ataxia telangiectasia, as well as FA cells were shown previously to develop chromatid breaks or gaps after x-irradiation during G_2 (3, 8, 9). The higher incidence of chromatid breaks in these previous studies probably resulted from the longer irradiation-fixation interval, 3–6 hr, than in the present study. During this longer interval, gaps could be converted into breaks (11).

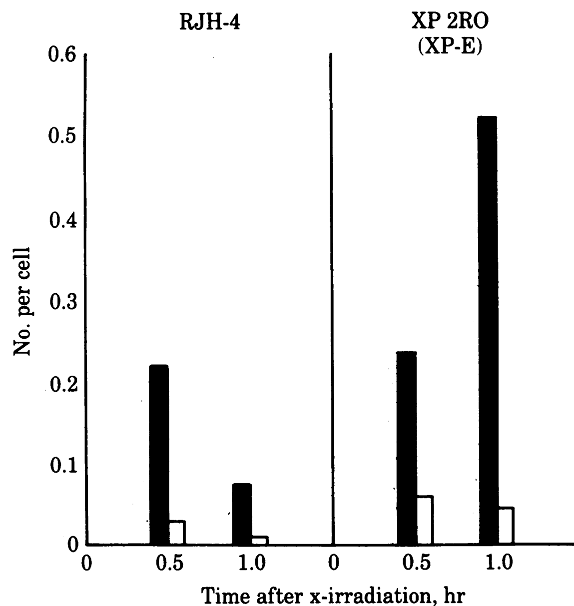

FIG. 1. Influence of time after x-irradiation (100 rad) on chromatid gaps (■) and breaks (□) in normal (RJH-4) and XP-E (XP 2RO) cells. For the 0.5-hr analysis, Colcemid was added for 0.5 hr immediately after irradiation; for the 1.0-hr analysis, the medium was renewed after irradiation and cultures were incubated for 1 hr with Colcemid added during the last 0.5 hr of incubation.

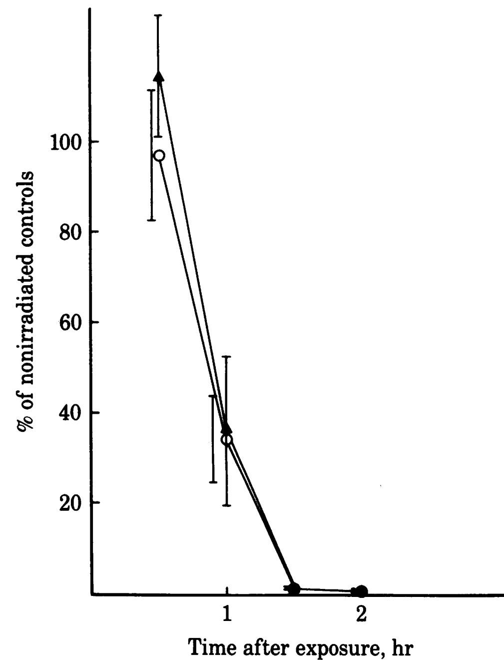

FIG. 2. Influence of x-irradiation (100 rad) on progression of G_2 cells into metaphase. Each determination is based on four irradiated and two nonirradiated control cultures and represents the ratio of the mean numbers of metaphase cells per culture in x-irradiated to nonirradiated controls. Bar indicates standard error of the ratio of the means. ○, RJH-4; ▲, HT 3252.

Considerable experimental evidence indicates that chromosomal aberrations result from unrepaired DNA damage (for review, see ref. 20). Each chromatid apparently contains a single continuous DNA double strand; therefore, a chromatid break must represent an unrepaired DNA double-strand break; chromatid gaps, especially prevalent in cells irradiated during late G_2 or early prophase (21), are thought to represent DNA single-strand breaks (11, 13). The increased incidence of x-ray-induced chromatid damage in the cells from cancer-prone individuals compared with normal cells could result from a deficiency in repair of the DNA lesions leading to chromatid gap or break formation. A second possibility is that the higher yield in the cancer-prone cells could result from a breakthrough of the x-ray-induced G_2 mitotic block, allowing a shorter time for DNA repair (22, 23). Our results show that the two cancer-prone lines tested exhibit a mitotic block at the same time after ir-

Table 3. Influence of 10 μM ara-C on chromatid damage induced in XP-A (XP 12BE, XP 25RO) and normal cells (KD) by x-irradiation during G_2

Cell line	Treatment	Cells studied, no.	Mean no. per cell: Chromatid gaps	Mean no. per cell: Chromatid breaks
XP 12BE	None	95	0.032	0.011
	50 rad	92	0.076	0.033
	50 rad + ara-C	172	0.070	0.023
XP 25RO	None	146	0.014	0.014
	50 rad	122	0.033	0.033
	50 rad + ara-C	200	0.035	0.020
KD	None	200	0	0.010
	ara-C	200	0	0.015
	50 rad	200	0.010	0.010
	50 rad + ara-C	185	0.124	0.065

radiation that normal cells do. Moreover, the incidence of chromatid gaps in the cancer-prone cells increased with a longer post-irradiation period, rather than decreased with more time for repair as in the normal cells. These findings implicate a deficiency in DNA repair as the cause of increased gap formation rather than a shorter period available for repair.

A third possibility is that the irradiation might induce a differential delay in the progression of cells through G_2-prophase, resulting in sampling of normal and cancer-prone cells at different stages of progression within 1.5 hr of metaphase. However, the present results show that the rates of progression of G_2 cells into metaphase after irradiation are similar in cell lines from a normal and a GS donor.

The high yield of chromatid aberrations, particularly gaps, induced in cells from cancer-prone individuals after G_2 x-irradiation are of particular interest in relation to DNA repair mechanisms. X-irradiation is known to produce DNA strand breaks and endonuclease-sensitive sites which probably represent base damage. Single-strand breaks could arise directly or indirectly from incomplete excision repair (15). In excision repair of damaged DNA bases, a discontinuity in the polynucleotide strand follows endonuclease incision. A subsequent defect in the repair of this discontinuity could lead to a chromatid gap. The present cytogenetic data on XP cells suggest a relationship between the formation of chromatid gaps seen at metaphase within 1.5 hr of x-irradiation and defective nucleotide excision repair. This relationship is supported by the following observations.

(*i*) XP-C and XP-E cells, known to be deficient in excision repair of UV-induced pyrimidine dimers, show an increased incidence of x-ray-induced chromatid gaps compared with excision-proficient XP-Va and normal cells. Furthermore, FA cells deficient in excision repair of γ-ray-induced DNA damage (7) also show an increased incidence of x-ray-induced chromatid gaps.

(*ii*) XP-A cells, which do not repair damage to DNA that requires endonucleolytic incision (24) and do not remove UV endonuclease-sensitive sites (25), do not show an increased incidence of x-ray-induced chromatid gaps. This observation suggests that endonuclease incision of DNA is requisite for chromatid gap formation after G_2 x-irradiation.

(*iii*) Addition of ara-C to cultures directly after x-irradiation significantly increased the incidence of chromatid gaps in normal cells but not in XP-A cells. ara-C is thought to inhibit excision repair of base damage (15, 26), and in the normal cells this base damage is apparently repaired by an ara-C sensitive nucleotide excision repair mechanism. The absence of an effect of ara-C on x-irradiated XP-A cells appears to result from their deficient endonucleolytic incision of DNA, an initial step in nucleotide excision repair and a probable requirement for chromatid gap formation.

No consistent or clear-cut dose–response with respect to x-ray-induced chromatid gaps was observed, in contrast to chromatid breaks. Although the damage leading to gap formation would be dose-related, chromatid gaps apparently result from a complex multistep excision repair process. The absence of a clear-cut dose–response could result from a rate-limiting step in this repair process, particularly in endonucleolytic incision of DNA. Thus, in spite of the larger number of lesions induced by exposure to 100 rad, compared to 50 or 25 rad, only a limited number independent of total lesions might be excised in the cells at any one time. Alternatively, only a limited number of damaged sites might be accessible to repair enzymes because of the chromatin configuration prior to and during prophase.

It appears that all of these cell lines from cancer-prone individuals, except XP-Va and XP-A cells, are deficient in excision repair of x-ray-induced DNA damage inflicted during G_2 phase and manifested as chromatid gaps at the subsequent metaphase. Although proficient in excision repair of UV-induced dimers, XP-Va cells show a marginally significant increase in x-ray-induced chromatid breaks compared with normal cells; these could result from deficient repair of DNA strand breaks. The deficiency in G_2-prophase repair of post-replicated DNA manifested as chromatid gaps is probably related to DNA gap filling and deficient polymerase or ligase activity. A significant decrease in DNA ligase activity and deficient exonuclease activity have been reported in FA cells (27, 28). It has also been shown that in ataxia telangiectasia cells the enzyme that enhances activity of DNA polymerase I is deficient (29).

A defect in DNA repair in all of these cancer-prone cells has not been reported in previous studies using biochemical methods or autoradiography of asynchronous cell populations, which consist primarily of G_1 and S phase cells. We also observed no difference between cells from normal and cancer-prone individuals with respect to chromatid damage in cells exposed to fluorescent light during G_1 and S phases; in contrast, cells from these cancer-prone individuals, except XP-A, showed a significant increase in chromatid damage, not seen in normal cells, when exposed to fluorescent light during late S-G_2 (unpublished data). Exposure to fluorescent light, like x-irradiation, produces chromatid damage through the intracellular generation of H_2O_2 and the derivative free hydroxyl radical (30).

In spite of the diverse pathologic characteristics associated with these genetic disorders, they all predispose the individual to a high risk of cancer and they all are apparently deficient in some step of DNA repair, predominantly excision repair operative during G_2-prophase period of the cell cycle.

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