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Expression of the *E.coli* fpg gene in mammalian cells reduces the mutagenicity of γ-rays

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ABSTRACT

The *E.coli* fpg gene encodes the formamido-pyrimidine-DNA-glycosylase (FPG protein) which specifically removes the formamido-pyrimidine and C8-oxoGuanine residues from γ-irradiated DNA. The fpg gene was ligated in the psV2 vector and transfected into the Chinese hamster CHO and V-79 cells. The transfected cells expressed a formamido-pyrimidine-DNA-glycosylase activity 30 to 40-fold over the constitutive level. The resistance of CHO and V-79 cells to the lethal effect of γ-rays was similar in control and transfected cells. Furthermore CHO cells expressing the fpg gene had the same resistance to the lethal effect of hydrogen peroxide as control cells. However,the sensitivity to the mutagenic effect of γ-rays, measured as 6-thioguanine resistance, decreased both in CHO and V-79 transfected cells. Since the lethal effect of γ-rays was not modified in cells overproducing the FPG protein, the results suggest that this protein protects the cells against the mutagenic lesions formed by ionizing radiations, and among them C8-oxoguanine.

INTRODUCTION

Free radicals can be generated *in vivo* from endogenous (e.g. oxidant enzymes, phagocytic cells) or exogenous sources (e.g. redox-cyclic drugs, ionizing radiations) and result in damage to cell DNA. In the case of ionizing radiations, DNA damage is produced by both direct and indirect effects, and the formation of free radicals, especially hydroxyl radicals $OH^{\cdot}$, results in a variety of DNA lesions, including DNA breaks, bases and sugar modifications, as well as DNA–protein crosslinks (1). Numerous modified bases have been characterized after γ-irradiation of isolated DNA, isolated chromatin or cells (2). They have been identified in hepatic chromatin of mice upon whole body γ-irradiation (3). The type of the products and their quantities depend on the radical environment and the presence of oxygen. Among the modified bases formed in γ-irradiated chromatin, C8-oxoGua is the most abundant (4).

C8-oxoGua residues seem to be moderately cytotoxic in bacterial systems (5), but different experiments have shown the mutagenic properties of these residues in bacteria, as they have the ability to generate G→T transversions during replication in *E.coli* (5–7). In mammalian cells, treatment with visible light in the presence of riboflavine results in the formation of C8-oxoGua in DNA and in an increased mutation frequency (8), suggesting the mutagenicity of these lesions. The use of a single-stranded shuttle vector containing a C8-oxoGua residue also showed G→T transversions in mammalian cells (9).

In *E.coli*, DNA damage caused by free radicals and other oxygen-derived species are repaired by the formamidopyrimidine-DNA-glycosylase (FPG protein) (10), which possesses an associated β-lyase activity that nicks DNA at apurinic/apyrimidinic sites (11) and a dRPase activity (12). Among thirteen modified products detected by gas chromatography/mass spectrometry in γ-irradiated DNA, the FPG protein significantly excises FaPyAde, FaPyGua, C8-oxoGua and a detectable amount of C8-oxoAde (13). A similar glycosylase activity, excising FaPyGua (14) and C8-oxoGua (15), also exists in mammalian cells, although it has been shown that C8-oxoGua could also be excised by an endonucleolytic activity (16).

No mammalian cells devoid of FPG activity have been described, that would allow to test the role of unrepaired C8-oxoGua in toxicity and mutagenicity. However, we have previously shown that expression of *E.coli*, or mammalian DNA sequences, expressing 3-methyladenine-DNA-glycosylase activity, were able to increase the cell resistance to alkylating agents (17). Therefore in order to identify the possible biological consequences of unrepaired oxydized purines in the DNA of γ-irradiated cells, we have constructed a plasmid encoding the *E.coli* fpg gene. The bacterial gene was expresed in CHO and V-79 cells.

In this paper we show that the cells which express the bacterial FPG protein have an increased resistance to the mutagenic effect of ionizing radiations.

MATERIALS AND METHODS

Plasmid construction

The FPG 220 plasmid, carrying the *E.coli* fpg gene (10) was provided by Dr S.Boiteux. The coding sequence of the gene (809 bp) was excized by *Hin*dIII and *Pvu*II then purified by gel electrophoresis. It was ligated in the *Hin*dIII site of the psV2-neo vector. Plasmid with the insert in the correct orientation was called psV2-FPG.

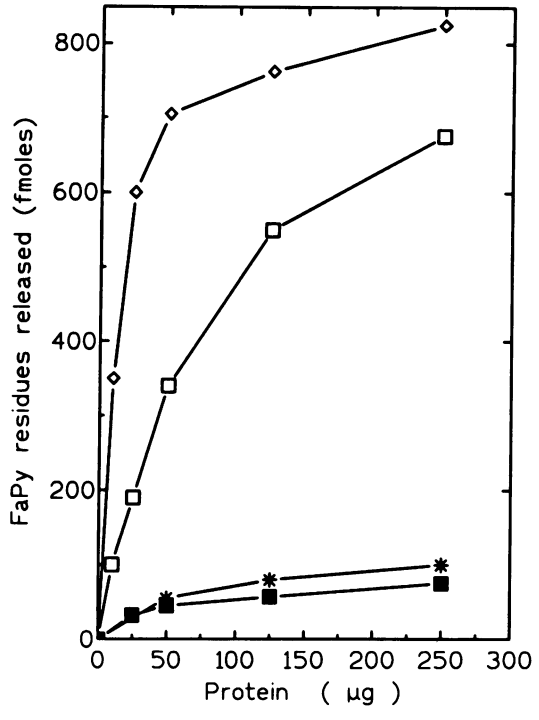

Figure 1. FaPy-DNA-glycosylase activity in CHO cells transfected with the psV2-FPG plasmid. Increasing amounts of cell extracts were incubated for 30 min at 37°C with [^{3}H]FaPy-poly(dG.dC). For details see Materials and Methods. Control cells (■); transfected cells, clone 1 (□) and clone 6 (◇); clone 6 cells incubated for 20 min at 37°C with *E.coli* FPG protein antibodies before measuring the glycoslase activity (*).

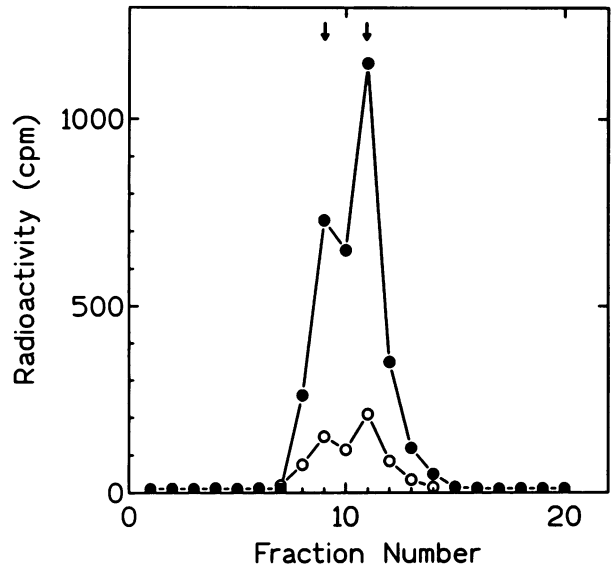

Figure 2. HPLC analysis of the reaction product. Cell extracts were incubated for 30 min at 37°C with [^{3}H]FaPy-poly(dG.dC). After ethanol precipitation, the supernatants were analysed by HPLC. Extracts from control (○) or transfected (●) CHO cells.The arrows indicate the position of the two FaPy rotamers.

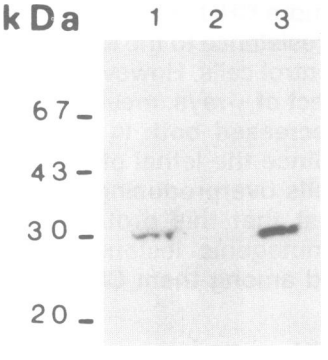

Figure 3. Immunoblot of CHO cells extracts expressing or not the FPG protein. Proteins were separated by gel electrophoresis and transferred to nitrocellulose. The membrane was incubated with rabbit antiserum raised against the purified FPG protein. lane 1: 200 µg of proteins from transfected cells. lane 2: 200 µg of proteins from control cells. lane 3: 50 ng of purified *E.coli* FPG protein.

Cell culture and transfection

CHO and V79 cells were grown in Dulcecco's medium supplemented with 5% fetal calf serum and 5% horse serum, in a 5% CO_2 humidified atmosphere. The psV2-FPG plasmid was linearized by treatment with *Eco*RI and was introduced in the cells by electroporation, using a Bio-Rad gene Pulser Apparatus, as described (17). The transfected cells were grown in G418 containing medium (750 µg/ml) until appearance of clones.

FaPy-DNA-glycosylase activity determination

Cells were harvested by trypsinization and suspended (2×10^8 cells/ml) in a buffer containing 70 mM Hepes, 100 mM KCl, 2 mM EDTA, 1 mM DTT and 10% glycerol. They were disrupted by sonication at 0°C in the presence of proteases inhibitors (leupeptin, aprotinin and antipain, 2 µg/ml each) and cell debris were removed by centrifugation (10 000 *g*, 5 min, 4°C). Increasing amounts of cell extracts were incubated, in a final volume of 100 µl, with [^{3}H]FaPy-poly dG.dC prepared as previously described (10). After 30 min at 37°C, the radioactivity present in the ethanol-soluble fraction was quantitated by scintillation spectroscopy. The presence of FaPy residues in the ethanol-soluble fractions was checked by HPLC analysis after addition of authentic markers, using a C18-µBondapack column eluting with 50 mM ammonium phosphate containing 5% methanol.

Survival and mutagenicity measurements

Exponentially growing cells were irradiated with a ^{60}Co γ-ray source at a dose rate of 1 Gy/min at room temperature. Incubations with H_2O_2 were done in serum free Dulbecco's medium for 30 min at 37°C, as already described (18). Cells were rinsed, trypsinized and aliquots of cell suspension were cultured until appearance of clones.

For the mutagenicity determination, cells were treated as described above. γ-irradiated cells were grown in fresh medium for 7 to 10 days to allow expression of the mutant phenotype. They were then subcultured in normal medium for survival measurement or in the presence of 6-thioguanine (2.5 µg/ml) for mutation frequency determination (18).

Immunoblotting

The rabbit antisera against the purified FPG protein was a gift from Dr J.Laval. SDS−PAGE was carried out as described (19). Immunoblotting and detection were done using the ECL kit (Amersham) according to the instructions of the manufacturer.

Table 1. FaPy-DNA-glycosylase activity and radiosensitivity in control and transfected cells

Cell Line	FPG activity*	Do values (Gy)
CHO	0.20	1.44 ± 0.036
CHO+psV2-FPG	7.88	1.46 ± 0.045
V79	0.22	2.27 ± 0.025
V79+psV2-FPG	7.50	2.35 ± 0.030

*pmoles of FaPy residues removed in 30 min at 37°C by 1 mg of proteins. The Do values were calculated from data of the survival curves and are the mean value ± S.D. of three separate experiments.

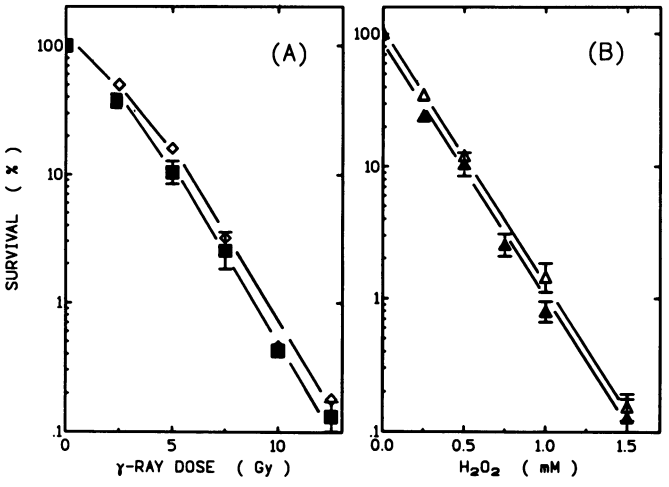

Figure 4. Survival curves for γ-irradiated or H_2O_2-treated cells. (**A**) Survival of γ-irradiated control (■) and transfected (◇) CHO cells. (**B**) survival of cells incubated for 30 min with H_2O_2: control (▲) and transfected (△) CHO cells. Results are the mean value ± S.D. of three separate experiments.

RESULTS

Expression of FaPy-DNA-glycosylase in transfected cells

Following transfection of CHO cells with the psV2-FPG plasmid, several G418 resistant clones were isolated. They expressed different levels of FaPy-DNA-glycosylase activity. For instance, clone 1 and clone 6 expressed an activity which was about 25 and 40-fold higher than the normal level measured in CHO cells, respectively (Fig. 1). HPLC analysis of the reaction products (Fig. 2) shows that they cochromatography with the two rotamers of the FaPyGua residues (20). To ascertain that this enhanced activity was due to the expression of the bacterial gene, the cell extracts were preincubated for 20 min with antibodies raised against the *E.coli* FPG protein. This preincubation abolished the activity, showing that it is due to expression of the bacterial protein (Fig. 1). Proteins from clone 6 cells were analyzed by Western blot using polyclonal antisera raised against the purified bacterial FPG protein: the results also confirm the presence of the FPG protein in the transfected cells and show that the expressed protein has the same size than the bacterial one (Fig. 3). This increased activity was stable in the transfected cells grown in the absence of G418, indicating stable integration of the plasmid in the cellular genome.

After transfection with the psV2-FPG plasmid, V-79 cells expressing a FaPy-DNA-glycosylase activity about 40-fold above the control value, were selected (Table 1).

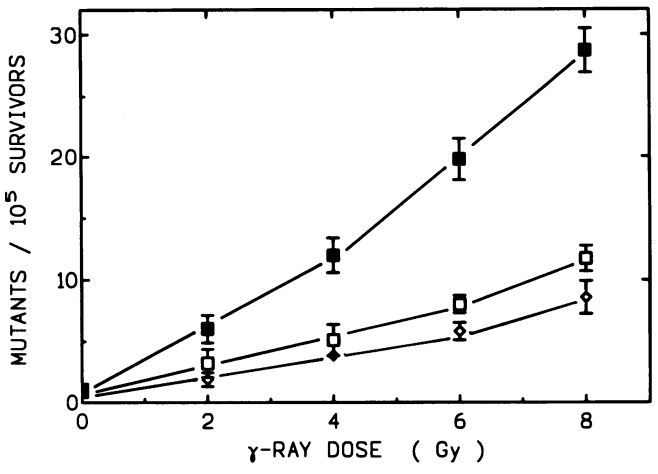

Figure 5. Mutation frequency in γ-irradiated cells. CHO cells were γ-irradiated then selected for 6-thioguanine resistance. Results are the mean value ± S.D. of three separate experiments. Control (■) and transfected CHO cells, clone 1 (□) and clone 6 (◇) cells.

Effect of FPG gene expression on cell sensitivity to γ-rays

The cytotoxic effect of γ-rays was measured in control and transfected CHO cells (Fig. 4A). CHO cells expressing the FPG protein have the same sensitivity to the lethal effect of radiations than the parental cells: neither the width of the shoulder nor the slope of the curve were significantly modified in the case of the transfected cells, showing that these cells cannot tolerate higher γ-ray doses than control cells. Data derived from the survival curves obtained with V-79 cells are summarized in Table 1: expression of the FPG protein did not modify the sensitivity of V-79 cells to the toxic effect of radiations.

Treatment with H_2O_2 in the presence of Fe^{3+} or Cu^{2+} generates hydroxyl radicals responsible of the formation of modified bases in DNA, and among them C8-oxoGua (21). Therefore we have measured the sensitivity of CHO cells expressing or not the FPG protein to the toxic effect of H_2O_2 (Fig. 4B): there was no significant difference in the sensitivity of both cell lines, as the Do doses were 0.150 and 0.157 mM, for control and transfected cells, respectively.

To determine whether oxydized purines are premutagenic lesions, the number of mutations in the HPRT locus was measured. The γ-ray induced mutation frequency was decreased in the transfected CHO cells compared to the control population and was lower in cells expressing the higher level of FPG protein (clone 6 cells) (Fig. 5). In the case of transfected V-79 cells, the number of mutants was only one third of that observed in control cells (data not shown), strongly suggesting that the lesions in mammalian cells. This decreased mutation frequency was not due to transfection artefact, because the same number of mutants was measured in irradiated non-transfected cells and in irradiated cells harbouring the psV2-neo vector.

DISCUSSION

As ionizing radiations produce a large number of lesions in DNA, it is difficult to ascertain the biological influence of a specific DNA damage. We have expressed the *E.coli* FPG activity in mammalian cells in order to determine the biological role of

oxydized purines and among them C8-oxoGua residues formed by γ-rays *in vivo*. The level of expression of the FPG protein in our transfected CHO or V-79 cells was high (about 30-fold over the constitutive value) compared to the expression of other repair proteins previously described in the literature, and thus allowed us to investigate the role of this protein in the cells.

The FPG protein removes the C8-oxoGua residues and other lesions (FaPyAde and FaPyGua) formed in lower amounts in irradiated chromatin (13). These damage do not seem to be involved in the cytotoxic effect of the radiation doses used in our experiments, as the survival of irradiated CHO and V-79 cells is not modified when these cells express a high level of FPG protein. The fact that expressing the FPG protein does not modify the CHO cells resistance to the toxic effect of H_2O_2 also suggests that C8-oxoGua is not cytotoxic.However we cannot exclude the hypothesis that C8-oxoGua residues are produced at a low level by γ-irradiation, compared to other types of toxic lesions, e.g. DNA double strand breaks, and hence that increasing the repair capacity for C8-oxoGua has no measurable effect on cell survival.

However our results show that expression of the fpg gene confers substantial resistance to the mutagenic effect of γ-rays, both in the case of CHO and V-79 cells. The protein encoded by the fpg gene is specific for the excision of modified purines, mainly C8-oxoGua lesions. It is also known that C8-oxoAde are much less mutagenic than C8-oxoGua residues in *E.coli* cells (22). Therefore the present results suggest that C8-oxoGua is mutagenic in mammalian cells, as it has been shown in other biological systems (7), and that the γ-ray induced damage which is reparable by the *E.coli* FPG protein is not a major lethal lesion in mammalian cells but rather a mutagenic lesion.

It has been suggested that oxygen-derived species might be implicated in the etiology of cancer (23) and elevated levels of modified bases, among them C8-oxoGua, have been measured in cancerous tissues (24). Therefore if C8-oxoGua residues are not totally removed, due to inefficient DNA repair, they could lead to mutations and therefore to cancer or age-related deseases (25).

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