Suppression of human DNA alkylation-repair defects by *Escherichia coli* DNA-repair genes

(*ada-alkB* operon/mammalian expression vector/Mer phenotype)

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ABSTRACT The *ada-alkB* operon protects *Escherichia coli* against the effects of many alkylating agents. We have subcloned it into the pSV2 mammalian expression vector to yield pSV2ada-alkB, and this plasmid has been introduced into Mer⁻ HeLa S3 cells, which are extremely sensitive to killing and induction of sister chromatid exchange by alkylating agents. One transformant (the S3-9 cell line) has several integrated copies of pSV2ada-alkB and was found to express a very high level of the *ada* gene product, the 39-kDa O⁶-methylguanine-DNA methyltransferase. S3-9 cells were found to have become resistant to killing and induction of sister chromatid exchange by two alkylating agents, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea. This shows that bacterial DNA alkylation-repair genes are able to suppress the alkylation-repair defects in human Mer⁻ cells.

DNA alkylation damage causes mutation and cell death in both human and bacterial cells. The probability that a particular cell will die or mutate is determined by the types of DNA damage incurred and by the cellular response to the damage. Comparisons of the repair of alkylated bases in populations of alkylation-sensitive and -resistant *Escherichia coli* have identified O⁶-methylguanine as the main mutagenic lesion that causes G-C to A-T transitions (1-3) and 3-methyladenine and 3-methylguanine as lesions that lead to cell death (4-6). It is unclear whether these lesions produce the same effects in human cells. Alkylation-sensitive (Mer⁻) human cell lines do exist (7, 8), but their phenotypes are complex and it has been difficult to use them to determine which lesions are mutagenic in human cells and which are lethal. Here we describe an approach in which we study killing and the induction of sister chromatid exchange (SCE) in Mer⁻ human cells that have integrated the bacterial *ada* and *alkB* genes and are producing the bacterial DNA methyltransferase.

The *E. coli ada* gene encodes a 39-kDa DNA methyltransferase that directly transfers methyl groups from O⁶-methylguanine, O⁴-methylthymine, and methyl phosphotriester DNA adducts to cysteine residues within the enzyme itself (9-12). The methyl groups remain covalently bound to the enzyme, and its DNA methyltransferase activity is consequently inactivated; i.e., the methyltransferase is a suicide enzyme (10). The *alkB* gene lies in the same operon as the *ada* gene (13). The specific function of the 27-kDa *alkB* gene product is not known, but it is known to be involved in the protection of bacteria from the lethal effects of alkylation damage (13, 14). We have subcloned the *ada-alkB* operon into a mammalian expression vector such that the operon comes under the control of the simian virus 40 (SV40) early

promoter, and we have transfected this construct into alkylation-sensitive human cells.

Human tumor cell lines fall into two phenotypically distinct groups. Cells of the Mer⁺ phenotype are resistant to alkylation-induced killing and SCE, support the growth of alkylated viruses (7, 15), and are able to repair O⁶-methylguanine (7, 8); cells of the Mer⁻ phenotype are exceedingly sensitive to cell killing and SCE induction, are poor hosts for alkylated viruses, and are unable to repair O⁶-methylguanine (7, 8, 15). Here we show that expression of the *E. coli ada-alkB* operon in Mer⁻ human cells restores their resistance to alkylating agents.

MATERIALS AND METHODS

Cells and Plasmids. HeLa S3 Mer⁻ cells (a gift from R. B. Setlow, Brookhaven National Laboratory) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, streptomycin (100 µg/ml), penicillin (100 units/ml), and L-glutamine (0.03%) and incubated in humidified 5% CO₂ at 37°C. G418-resistant transformants were grown in the presence of 200 µg of the aminoglycoside G418 (GIBCO) per ml. *E. coli* Y1089 (a gift from B. Meyer, Massachusetts Institute of Technology) were grown in rich medium. pSV2neo (16) and pSV2βg were gifts from P. Berg. Plasmid pGW2607, carrying the *ada-alkB* operon, was a gift from G. Walker and P. LeMotte (17). Plasmid DNA was purified by NaDodSO₄ lysis followed by banding in cesium chloride/ethidium bromide equilibrium gradients (18). The construction of pSV2ada-alkB is described in Fig. 1. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. *E. coli* HB101 was used as host.

Transfection and Selection of Transformants. HeLa S3 Mer⁻ cells were seeded at 10⁶ cells per 100-mm dish on day 1. On day 2, 2 µg of pSVada-alkB and 0.2 µg of pSV2neo were cotransfected into these cells by using the calcium phosphate technique for gene transfer (19). On day 3, the cells were shocked with 10% dimethyl sulfoxide for 30 min. Each plate of cells was split 1:4 on day 4. From day 6 to day 20, G418 (400 µg/ml) was included in the medium, and ten G418-resistant colonies were isolated by use of glass cloning rings. The transformants have been grown in medium with 200 µg of G418 per ml since that time.

DNA Methyltransferase Activity Gels. A radioactively alkylated *Micrococcus luteus* DNA substrate in which 95% of the radioactively labeled purines were in the form of O⁶-methylguanine was generated by the method of Karran *et al.* (3), using [³H]methylnitrosourea from Amersham (2.9 Ci/mmol; 1 Ci = 37 GBq); the specific activity was 130 cpm/µg of DNA. Extracts were made from cells grown in

FIG. 1. Construction of pSV2ada-alkB. kb, Kilobases.

three 850-cm² roller bottles; cells were harvested by scraping, washed twice in cold phosphate-buffered saline (25 mM potassium phosphate, pH 7.0/0.15 M NaCl/0.015 M sodium citrate), and resuspended at 5×10^7 per ml in 50 mM HEPES/KOH (pH 7.8) containing 10 mM dithiothreitol, 1 mM EDTA, and 5% (vol/vol) glycerol. Cells were disrupted by sonication, and the sonicate was centrifuged at $9000 \times g$ for 15 min. The supernatants were frozen in liquid nitrogen and stored at -70°C . Human cell extract (3 mg of protein) was incubated with about 15 μg of DNA substrate at 37°C for 15 min. The extract proteins were then subjected to NaDod-SO₄/12% polyacrylamide gel electrophoresis. The gel was cut into 2-mm slices, which were incubated overnight at 55°C in nonaqueous scintillation fluid containing 5% Protosol (New England Nuclear) and then assayed for tritium by liquid scintillation counting.

Southern Blot Procedures. Isolation of genomic DNA and Southern blot analysis were carried out as described (18). Genomic DNA (10 μg) was digested with the indicated restriction endonucleases and the products were separated by electrophoresis in a 1% agarose gel. Blotting of the DNA onto nitrocellulose was by passive diffusion. Two hundred nanograms of a 1.3-kb HindIII-BamHI fragment containing the *E. coli* *ada-alkB* operon (17) was labeled with ³²P by nick-translation and used as probe. The final filter wash was at high stringency (30 mM NaCl/3 mM sodium citrate at 58°C).

Cell Survival and SCE Determinations. Cell survival after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MeNNG) or *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) was estimated by colony-forming ability. For MeNNG killing, 100-mm tissue culture dishes were seeded with 500, 5000, or 50,000 cells in 10 ml of culture medium, and the appropriate amount of freshly diluted MeNNG was added immediately. MeNNG stock solutions (1 mg/ml) in 100 mM acetate

buffer (pH 5.0) were stored at -20°C for up to 1 year without any loss of potency; dilutions were made in 100 mM acetate buffer (pH 5.0). Since MeNNG has a reported half-life of about 15 min in tissue culture medium (16), the cells did not have to be washed after MeNNG addition. In the case of BCNU, the cells were allowed 24 hr to attach to the plates and BCNU exposure was for 1 hr, after which the medium was replaced. BCNU (Bristol Laboratories, Syracuse, NY; a gift from W. Henner) was first dissolved in ethanol and then diluted to 20 mM with water [the final solution was 13% (vol/vol) ethanol]. BCNU stock solutions were always made fresh. After 14 days of incubation, colonies were fixed in Carnoy's fixing solution (methanol/glacial acetic, 3:1) and stained in 5% Giemsa stain. The colony-forming efficiency was expressed as a percentage of the colonies at the zero dose.

For MeNNG-induced SCE measurements, 2×10^6 cells were seeded in 150-cm² flasks, MeNNG was added immediately, and the cells were cultured for two rounds of DNA replication (2 days) in complete medium containing 10 μM bromodeoxyuridine. In the case of BCNU-induced SCE measurements, 10^6 cells were seeded in 150-cm² flasks and allowed 24 hr to attach. The cells were then exposed to BCNU for 1 hr, the medium was replaced with fresh medium containing 10 μM bromodeoxyuridine, and the cells were cultured for two rounds of DNA replication. Two hours after the addition of 0.2 μM Colcemid, mitotic cells were collected by shaking, treated in hypotonic solution (0.2% KCl, 0.2% sodium citrate, 5% fetal bovine serum) at 37°C for 15 min, and then fixed in Carnoy's solution. To produce "harlequin" chromosomes, we used a modified fluorescence plus Giemsa technique (20). Slides were stained in Hoechst 33258 (5.0 $\mu\text{g}/\text{ml}$) for 20 min, mounted in 0.067 M Sorensen's buffer (Na₂HPO₄/KH₂PO₄, pH 6.8) with a coverslip, exposed to a General Electric 15-W blacklight (F15T8.BLB) at 55°C for about 5 min, rinsed, and stained in a 5% Giemsa solution. At least 20 cells in metaphase were counted per data point.

RESULTS

Construction of pSV2 Containing the *ada-alkB* Operon (Fig. 1). The pSV2 mammalian expression vector was designed to allow cloned genes to be bounded on their 5' side by a strong promoter [the simian virus 40 (SV40) early promoter] and on their 3' side by a splicing site [the small tumor (t) antigen intron] and a polyadenylation signal (16). The insertion of the *E. coli* *ada-alkB* operon into pSV2 is outlined in Fig. 1. The 2.2-kb HindIII-BamHI fragment containing the *ada-alkB* operon was purified from the plasmid pGW2607 (17). The pSV2 backbone was prepared from pSV2βg by removing the HindIII-Bgl II fragment that carries the rabbit β-globin gene. Ligation of the *E. coli* derived HindIII-BamHI fragment and the pSV2 backbone generated the plasmid pSV2ada-alkB.

Isolation of HeLa Mer⁻ Cell Transformants Expressing *E. coli* Methyltransferase Activity. HeLa S3 cells are phenotypically Mer⁻ (7, 21, 22), are extremely sensitive to alkylation-induced killing and SCE, and lack DNA methyltransferase activity. pSV2ada-alkB was cotransfected into HeLa S3 cells along with the plasmid pSV2neo, which carries the *E. coli* gene (*neo*) that makes mammalian cells resistant to the aminoglycoside G418 (16). Ten G418-resistant transformant cell lines were isolated (S3-1 through S3-10) and screened for the presence of *E. coli* DNA methyltransferase activity.

Since labeled methyl groups from the O⁶ position of guanine become covalently attached to methyltransferase as a consequence of the process of repair, it is possible to use this reaction to detect the presence of enzyme in cell extracts. In this way we detected a very high level of DNA methyltransferase activity in cell extracts of one (S3-9) of the ten G418-resistant transformants (Fig. 2B). These cells actually contain about 4 times as much DNA methyltransferase

FIG. 2. Analysis of *O*⁶-methylguanine-DNA methyltransferase activity of cell extracts. Cell extract (3 mg of protein) from *E. coli* Y1089 (A), S3-9 (B), and S3-7 (C) were incubated with 15 μ g of *O*⁶-[³H]methylguanine-containing DNA substrate and then subjected to NaDodSO₄/12% PAGE. The location of the ³H-labeled protein in the gel was determined by cutting the gel into 2-mm slices and eluting the protein for liquid scintillation counting. Slice 1 is the top of the gel. The DNA substrate did not migrate more than 4 mm into the gel (data not shown).

activity as the original Mer⁺ HeLa cells (American Type Culture Collection CCL 2) from which our Mer⁻ cells were descended (data not shown). In bacteria, the *ada* gene product is a 39-kDa protein which quickly breaks down in cell extracts to a 19-kDa fragment (see Fig. 2A and ref. 23). The methyltransferase in S3-9 cells is also 39 kDa and it too breaks down to a 19-kDa fragment, though not as quickly (Fig. 2). By way of confirmation, we have also detected a 39-kDa protein in S3-9 cells that reacts with antiserum that recognizes the bacterial DNA methyltransferase (data not shown). Since the function of the *alkB* gene is not known, and since we do not have antiserum directed against the *alkB* protein, we were unable to determine whether this gene, which lies downstream from the *ada* gene, is also expressed in S3-9 cells.

Southern blot analysis, using as probe a *Hind*III-*Bam*HI fragment containing the *ada-alkB* operon (17), showed that the S3-9 transformant contains more than one copy of the *ada* gene, which give rise to several *Hind*III fragments of different sizes (Fig. 3, lane 1). Some of these copies must exist as tandem repeats of the entire plasmid (i.e., must include both *ada* and *alkB*), because *Hind*III digestion of the genomic DNA produced a major band at 6.4 kb, which is the size of pSV2*ada-alkB* cleaved at its only *Hind*III site (Fig. 3, lane 1). Similarly, digestion with both *Hind*III and *Bam*HI produced the expected 3.0-kb fragment (that covers the 2.2-kb *ada-alkB* operon and 0.847 kb of the pSV2 backbone) plus some other bands (Fig. 3, lane 3).

Analysis of the Phenotype of S3-9 Cells. We compared the alkylation sensitivity of the methyltransferase-positive S3-9 and one of the methyltransferase-negative cell lines (S3-7, see Fig. 2). The sensitivities of these two cell lines to MeNNG- or BCNU-induced cell killing and SCE are shown in Figs. 4 and 5. Clearly the S3-9 cells, carrying the pSV2*ada-alkB* plasmid and expressing the *E. coli* DNA methyltransferase (and possibly also the *alkB* gene product), have become

FIG. 3. Southern blot analysis of HeLa S3 and S3-9 genomic DNA, using the *E. coli ada* gene as probe. Samples (10 μ g) of HeLa S3 and S3-9 genomic DNA were digested with either *Hind*III alone or *Hind*III plus *Bam*HI. Digestion products were separated by electrophoresis in a 1% agarose gel, transferred to nitrocellulose, and hybridized with the ³²P-labeled *Hind*III-*Bam*HI DNA fragment from pGW2607 that contains the entire *E. coli ada-alkB* operon (17). *Hind*III fragments of bacteriophage λ were used as size markers (shown at left in kb). Lanes 1 and 2: *Hind*III-digested S3-9 and HeLa S3 DNAs, respectively. Lanes 3 and 4: *Hind*III/*Bam*HI-digested S3-9 and HeLa S3 DNAs, respectively.

resistant to cell killing and SCE induction by both MeNNG and BCNU.

DISCUSSION

We have set out to determine whether it is feasible to suppress the alkylation sensitivities of Mer⁻ human cells with bacterial DNA repair genes. During the course of this work, others reported (24) that the bacterial *ada* gene can be at least transiently expressed in monkey cells, but it was not shown whether this ever led to a stable alteration in cell phenotype. We report here that resistance to alkylating agents is restored to HeLa Mer⁻ cells by the genomic integration of a mammalian expression vector carrying the *E. coli ada-alkB* operon. At least one gene in this operon, the *ada* gene, is expressed in the alkylation-resistant S3-9 transformant cell line, and its product (a 39-kDa DNA methyltransferase)

FIG. 4. Alkylation-induced cell killing in S3-7 and S3-9 cells. The colony-forming ability of S3-7 cells (○) and S3-9 cells (●) was measured after treatment with either MeNNG (A) or BCNU (B).

Fig. 5. Alkylation-induced SCE in S3-7 and S3-9 cells. The frequency of SCEs induced in S3-7 cells (○) and S3-9 cells (●) by MeNNG (A) or BCNU (B) was measured as described in *Materials and Methods*.

appears to be stable in these cells. The intact *E. coli* DNA methyltransferase has two active sites; one site receives methyl groups from *O*⁶-methylguanine and *O*⁴-methylthymine, and the other site receives methyl groups from methyl phosphotriester lesions (9–12). The function of the *alkB*-encoded protein is not known. From the observed suppression, it seems that S3-9 cells have become better able to repair whatever lesions are responsible for cell killing and the production of SCEs in human cells.

BCNU forms lesions at the *O*⁶ position of guanine that can be repaired by the bacterial DNA methyltransferase before the lesions undergo a further reaction to create lethal DNA interstrand crosslinks (25). The sensitivity of Mer[−] cells to killing by BCNU has thus been attributed to their lack of methyltransferase activity (26). We were therefore not surprised to find that S3-9 cells are resistant to the lethal effects of BCNU, since these cells express a very high level of DNA methyltransferase activity. Since SCEs often correlate with cell killing (27), we were also not surprised that S3-9 cells are resistant to the SCE-inducing effects of BCNU.

However, several recent studies (28–31) have indicated that the human DNA methyltransferase protects cells against the induction of mutation by MeNNG but does not protect cells against its lethal effects. Thus the specific depletion of DNA methyltransferase activity from Mer⁺ human cells (by growth in the presence of free *O*⁶-methylguanine) sensitizes them to alkylation mutagenesis but not to killing (28–30). The reason that S3-9 cells have become exceedingly resistant to killing by MeNNG might be that they have also acquired the *alkB* gene product and that it is this that confers the resistance to alkylation-induced killing. It should be possible to alter the pSV2ada-*alkB* plasmid so that only the *alkB* gene or only the *ada* gene is expressed; this would allow us to determine whether the lesions responsible for mammalian cell killing and SCEs are the targets for the *alkB* gene product (which are at present unknown even in bacteria) or the *ada* gene product. In addition, the *ada* gene can be further dissected to yield enzymes that have only one active site and are thus able to repair only methyl phosphotriester lesions or only *O*⁶-methylguanine and *O*⁴-methylthymine lesions (32).

It was recently shown (33) that the bacteriophage T4 *denV* gene, which codes for a pyrimidine dimer-specific repair endonuclease, can confer partial resistance to the lethal effects of ultraviolet light upon Chinese hamster ovary cells. Whether complementations like that and the kind we report here can be used to confer a useful level of extra resistance on the bone marrow cells of cancer patients receiving chemotherapy remains to be determined.

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