Evidence for reactive oxygen species inducing mutations in mammalian cells

(radiation hypersensitivity/deletion assay/CHO/hprt/AS52 gpt)

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ABSTRACT We have studied the mutagenicity (by selecting for mutants resistant to 6-thioguanine) and cytotoxicity (by determining cellular cloning efficiency) of physical and chemical agents in Chinese hamster ovary (CHO) cells, clone CHO-K1-BH4 (K1-BH4), and its radiation-hypersensitive transformant, AS52. AS52 cells contain a single functional copy of a bacterial gene, the xanthine/guanine phosphoribosyltransferase (gpt), the bacterial equivalent of the mammalian gene hprt, for hypoxanthine/guanine phosphoribosyltransferase-deficient CHO cells (X3/5) that had been transformed by the plasmid vector pSV2gpt. One of the transformants, AS52, carries a single copy of the gpt gene stably integrated into the high molecular weight host DNA (12). X-irradiation is equitoxic to AS52 and its parental CHO cells, clone CHO-K1-BH4 (K1-BH4); however, x-ray is 10 times more mutagenic to AS52 cells than to K1-BH4 cells. Our earlier studies using Southern blot analysis showed that x-irradiation produces mostly or exclusively deletion mutations in both cell types. If reactive oxygen species mediate the mutagenic effects of radiations and chemicals, then radiomimetic compounds such as streptonigrin and bleomycin, which exert their biological effects via reactive oxygen species, and oxidizing compounds such as potassium superoxide and hydrogen peroxide should elicit a similar differential mutagenic response in both cell types. On the other hand, agents such as ethyl methanesulfonate, ICR 191, and UV light, which do not produce reactive oxygen species, should not elicit differential mutagenicity. Our results fulfill such predictions. The apparent hypermutability of AS52 cells probably results from a higher recovery of mutilocus deletion mutants in AS52 cells than in K1-BH4 cells, rather than a higher yield of induced mutants.

It has been proposed that the adverse biological effects of radiation are mediated through reactive oxygen species that are produced during reactions between radiation and the biological system (1). Reactive oxygen species are being increasingly implicated in the initiation and progression of various diseases and in the toxic action of numerous chemicals (2-4).

Since the discovery of the mutagenic effects of x-rays in Drosophila (5), the genetic effects of radiation on a wide spectrum of biological endpoints in a variety of species and experimental systems have been extensively studied (6). The demonstration that chemical mutagens could induce mammalian somatic cell variants with altered phenotypes has generated considerable interest in utilizing mutational assays in rodent and human cells to study the mechanism of gene mutation and to determine the mutagenic activity of radiation and environmental chemicals (7, 8).

We have used Chinese hamster ovary (CHO) cells to study mutations induced by chemical and physical agents at the hypoxanthine/guanine phosphoribosyltransferase locus (hprt) (9, 10). Our early studies indicated that x-irradiation is weakly mutagenic in this assay; the shape of the dose–response curve could not be adequately defined due to the weak mutagenicity (11).

Recently, we developed a system to study mutations that affect expression of the xanthine/guanine phosphoribosyltransferase gene (gpt), the bacterial equivalent of the mammalian gene hprt, in hypoxanthine/guanine phosphoribosyltransferase-deficient CHO cells (X3/5) that had been transformed by the plasmid vector pSV2gpt. One of the transformants, AS52, carries a single copy of the gpt gene stably integrated into the high molecular weight host DNA (12). X-irradiation is equitoxic to AS52 and its parental CHO cells, clone CHO-K1-BH4 (K1-BH4); however, x-ray is 10 times more mutagenic to AS52 cells than to CHO cells (12, 13). Thus, AS52 cells are hypersensitive to x-ray-induced mutations.

Chemicals that exhibit biological effects similar to those of radiation have been referred to as radiomimetic chemicals. If reactive oxygen species were to mediate the biological effects of both radiomimetic chemicals and radiation (Fig. 1), chemilumines such as streptonigrin (14) and bleomycin (15), which exert their effects via reactive oxygen species, could be expected to be equitoxic to K1-BH4 and AS52 cells, but more mutagenic in AS52 cells than in K1-BH4 cells. Likewise, highly reactive oxidizing agents such as superoxide and hydrogen peroxide, which are reactive oxygen species, should be more mutagenic in AS52 cells and equitoxic to both cell types. Here we present evidence for the role of reactive oxygen species in the mutagenic and toxic action in mammalian cells.

MATERIALS AND METHODS

We used subclone K1-BH4 of the CHO-K1 cell line (9) and AS52 cells (12) for all of the experiments. Female Chinese hamsters possess 22 chromosomes with two X chromosomes (16); however, K1-BH4 cells have a model chromosome number of 20 with only one X chromosome; its short arm replicates earlier than the long arm. J. R. San Sebastian and A.W.H., unpublished data). AS52 cells contain a single, functional copy of Escherichia coli gpt gene stably integrated into the genome of clone X3/5, an x-ray-induced hprt deletion mutant of K1-BH4 cells (12, 17).

We used a standard procedure to determine the cytotoxicity and mutagenicity affected by physical and chemical agents (11, 17). Streptonigrin was a gift from L. G. Littlefield of Oak Ridge Associated Universities, who obtained it from J. Berman of Microbiological Associates.

Abbreviations: K1-BH4, CHO-K1-BH4 subclone of Chinese hamster ovary cell line K1; EtMes, ethyl methanesulfonate; TG, 6-thioguanine, TG', TG resistant.

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RESULTS

Our early studies of radiation mutagenesis with K1-BH4 cells showed that x-ray is weakly mutagenic, causing a dose-dependent increase of mutations to TG resistance in the range of 200–800 rads (11–13). Although x-irradiation is equitoxic to both K1-BH4 and AS52 cells, it is ≈10 times more mutagenic to AS52 cells than to K1-BH4 cells (12, 13).

The pattern of x-ray-induced differential mutagenic response between K1-BH4 and AS52 cells was produced also by irradiation with neutrons. Neutron-irradiation resulted in nearly identical toxicity to both cell types; however, neutron-irradiation was ≈10 times more mutagenic to AS52 cells than to K1-BH4 cells (Fig. 2).

If reactive oxygen species were to mediate the mutagenic effects of radiation such as x- and neutron-irradiation, then radiomimetic chemicals such as streptonigrin, an antitumor drug shown to produce superoxide and/or hydroxyl radicals leading to chromosome aberrations and DNA strand scissions (14), should elicit a similar differential mutagenic response in these two cell types (Fig. 1). Our experiments showed that streptonigrin is much more mutagenic to AS52 cells than to K1-BH4 cells and is nearly equitoxic to both cells (Fig. 3). A similar pattern was found in experiments with another radiomimetic chemical, bleomycin (from Bristol Laboratories, Syracuse, NY), which produces reactive oxygen species as its mechanism of action (refs. 15 and 20; D.S.K. and A.W.H., unpublished data).

If the mutagenic effects of both radiation and radiomimetic chemicals are mediated through reactive oxygen species,
potassium superoxide, a highly active oxidizing compound, should be highly mutagenic to AS52 cells. We found that potassium superoxide exhibits similar toxicity to both cell types. Potassium superoxide was extremely mutagenic to AS52 cells but did not produce significant mutations in K1-BH4 cells (Fig. 4). A similar pattern of "identical toxicity and differential mutagenicity" was found for another oxidizing chemical, hydrogen peroxide (from Fisher Scientific) (L.R. and A.W.H., unpublished data).

These experiments with AS52 and K1-BH4 cells provided evidence for the direct role of reactive oxygen species in the induction of gene mutations in mammalian cells. The high mutagenic response of AS52 cells to radiation and radiomimetic chemicals suggests that these cells are sensitive enough to be used to define the shape of a dose–response relationship (21) from future experiments with a large number of doses and different irradiation dose rates. The low mutagenic response of K1-BH4 cells may imply that reactive oxygen species induce primarily deletions resulting in a reduced mutant recovery in this cell type (see Discussion).

**DISCUSSION**

Our studies demonstrate that physical and chemical agents that produce reactive oxygen species as their mechanism of action, including two types of radiation (neutrons and x-rays), two radiomimetic chemicals (streptonigrin and bleomycin), and two oxidants (potassium superoxide and hydrogen peroxide), are all much more mutagenic to AS52 cells than to K1-BH4 cells (Figs. 2–4). By contrast, UV light (13), ethyl methanesulfonate (EtMes) and ICR 191 (22), none of which produces reactive oxygen species, are not more mutagenic to AS52 cells than to K1-BH4 cells (Table 1). These findings support the view that reactive oxygen species induce gene mutations in mammalian cells.

We have suggested (12) that the difference in the slope of the induced mutation curves between the two cell types when irradiated with x-rays may reflect, in part, differences in the target size or DNA sequence between the hprt gene in K1-BH4 cells and the gpt gene in AS52 cells. X-irradiation has been found to induce a higher mutation frequency in gpt-
transformed V79 cells than in their parental V79 cells (23), and potassium superoxide has induced TG' mutants in another CHO cell line (24).

It is noteworthy that, like the parental K1-BH4 cells, AS52 cells have a stable and low spontaneous mutation frequency (12, 17). This is in contrast to our other classes of gpt-transformed CHO cells (17) and similarly transformed V79 cells (23), all of which exhibit a relatively high spontaneous mutation frequency.

If the hypersensitivity of the gpt gene in AS52 cells to radiation, radiomimetic chemicals, and oxidizing compounds is attributed to the integration of the bacterial gene into the mammalian cell chromosome, which is known to be relatively unstable in some instances (17, 23), AS52 cells should be indiscriminately sensitive to any mutagen, including those whose mode of action is unrelated to the production of reactive oxygen species. However, chemical mutagens such as EtMes, ICR 191, and UV-irradiation were found to elicit comparable cytotoxic and mutagenic responses in both K1-BH4 and AS52 cells, suggesting that the bacterial origin of the locus is not significant in eliciting the mutagenicity of these agents (refs. 13 and 22; Table 1). Agents known to produce reactive oxygen species, including radiation (x-rays and neutrons), radiomimetic chemicals (streptonigrin and bleomycin), and oxidizing compounds (potassium superoxide and hydrogen peroxide), are highly mutagenic to AS52 but not to K1-BH4 cells (Figs. 2–4; Table 1). These observations suggest that reactive oxygen species could be used as a chemical dosimeter to correlate with the biological effects expected from a biological dosimeter, the AS52 cell strain, when these cells are exposed to various environmental physical and chemical agents (Fig. 1).

Using Southern blot analyses, we have shown that x-rays induced predominantly, if not exclusively, deletion mutations; however, EtMes, ICR 191, and UV light caused no detectable change in the DNA sequence (obtained by Southern blot analysis) of either hprt or gpt in K1-BH4 and AS52 cells, respectively (13, 22). These results suggest that radiomimetic chemicals and oxidizing compounds, like radiation, induce predominantly deletion mutations, while EtMes, ICR-191, and UV light cause mainly point mutations in both genes. This is consistent with the observations that radiation and radiomimetic chemicals are highly effective in causing chromosomal aberrations and that EtMes and ICR 191 are known to induce primarily missense and frame-shift mutations, respectively (25, 26).

The purine-salvage pathway enzymes hypoxanthine/guanine and xanthine/guanine phosphoribosyltransferases, the gene products of hprt and gpt genes in K1-BH4 and AS52 cells, respectively, are not essential for cell survival. All of the mutagens studied produce a similar toxic response in these cell lines, which only differ in the two genetic markers (Figs. 2–4). In mammals, the hprt gene is localized on the X chromosome, where the genes exist in a hemizygous state. Using the TG-selection system, a deletion conferred on the hprt gene per se might not affect cellular survival; however, a deletion larger than the hprt gene could involve genes vital to survival. Since x-ray induces predominantly deletion mutations (13), the low mutagenic activity of x-irradiation in K1-BH4 cells is likely due to lethal events associated with multiple-locus deletions that reduce the number of recoverable mutants under selection conditions. By inference from experiments with x-rays, the low mutagenic response of radiomimetic chemicals and oxidizing compounds may also be due to low mutant recovery rather than reduced mutation induction.

Although x-rays and neutrons induced more mutants in AS52 cells (due to a higher mutant recovery) than in K1-BH4 cells, these agents did in fact induce a low but detectable level of mutants in the latter cell line (Fig. 2). On the other hand, streptonigrin and potassium superoxide do not induce a significant frequency in K1-BH4 cells (Figs. 3 and 4). This finding suggests that the spectrum of mutations induced by these chemicals may differ from that induced by radiation. It is possible that in K1-BH4 cells, reactive oxygen species produced by radiation and chemicals cause DNA strand breaks leading to deletion mutations, which are unlikely to be recoverable, and that base damage, another mode of action known for radiation (6, 13), resulted in some recoverable mutants. Thus, some mutants are induced and recovered by radiation, while an insignificant quantity of mutants are induced by these chemicals in K1-BH4 cells.

In AS52 cells, the gpt gene may be stably incorporated into an autosome. The genes localized in autosomes probably exist in the diploid state. Even a multiple-locus deletion could produce a hemizygous state in the vital genes that may flank the gpt gene (in contrast to a loss of these vital genes with a deletion larger than the hprt gene in K1-BH4 cells), leading to a higher probability of recovering induced TG' mutants. Although x-irradiation should be equally efficient in producing TG' mutants in both cell types, the lower probability of deletion-associated lethality in AS52 cells permits higher recovery of mutants than from K1-BH4 cells. The high mutagenic response of AS52 cells to radiomimetic chemicals and oxidizing compounds could be due to a higher recovery of TG' mutants in AS52 cells than in K1-BH4 cells.

We have shown in our paper that six physical and chemical agents that exert their biological effects via reactive oxygen species display differential mutagenicity in K1-BH4 and AS52 cells, and three agents that do not produce reactive oxygen species do not exhibit a differential effect. This is still short of proving that reactive oxygen species per se are inducing gene mutations in mammalian cells, since, in theory, there may be one or more other mutagenic principles common to the six agents that produce reactive oxygen species—principles that are not possessed by the three agents that do not produce reactive oxygen species. In the absence of any evidence as to what such mutagenic principles other than reactive oxygen species might be, we believe we are justified
in identifying reactive oxygen species as the most probable cause of mutation.

The differential mutability of K1-BH4 and AS52 cells to radiation and radiomimetic chemicals should provide a diagnostic tool for detecting and quantifying environmental agents whose primary mode of action is mediated through reactive oxygen species, which in turn cause deletion mutations through oxidative DNA damage. If a test agent mimics the mutagenic response seen with x-irradiation in K1-BH4 and AS52 cells, it could be suspected of being capable of inducing deletions and/or chromosomal breakage. On the other hand, agents that induce deletions by whatever mechanism would induce more mutants at the gpt locus in AS52 cells than at the hprt locus in K1-BH4 cells. One could also predict that radiations, radiomimetic chemicals, and oxidizing compounds would appear to be more mutagenic (i.e., more mutants recoverable) to hemizygous genetic markers localized on autosomes than on the X chromosome in various mammalian cell mutational assays (27, 28). These predictions have been fulfilled recently by a series of experiments performed with mouse L5178 cells (D. M. DeMarini and M. Moore, personal communications).

It has been suggested that a mechanism of spontaneous mutation involves DNA base-pair changes (29). However, spontaneous deletion mutations have been observed with a relatively high frequency in the lac gene of E. coli (30), the histidiner operon of Salmonella typhimurium (31), the human β-globin gene (32), the gpt gene of AS52 cells (17), and the hprt gene from human T lymphocytes (33, 34). One may reasonably assume that certain naturally occurring or apparently innocuous compounds can be reduced by intracellular reductases and, in turn, reduce oxygen to superoxide anion radical and other reactive oxygen species via a redox cycling mechanism (35), leading ultimately to the spontaneous occurrence of (deletion) mutations (35, 36).

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7. Hsie, A. W., O'Neil, J. P. & McElheny, V. K. (1979) Mam-