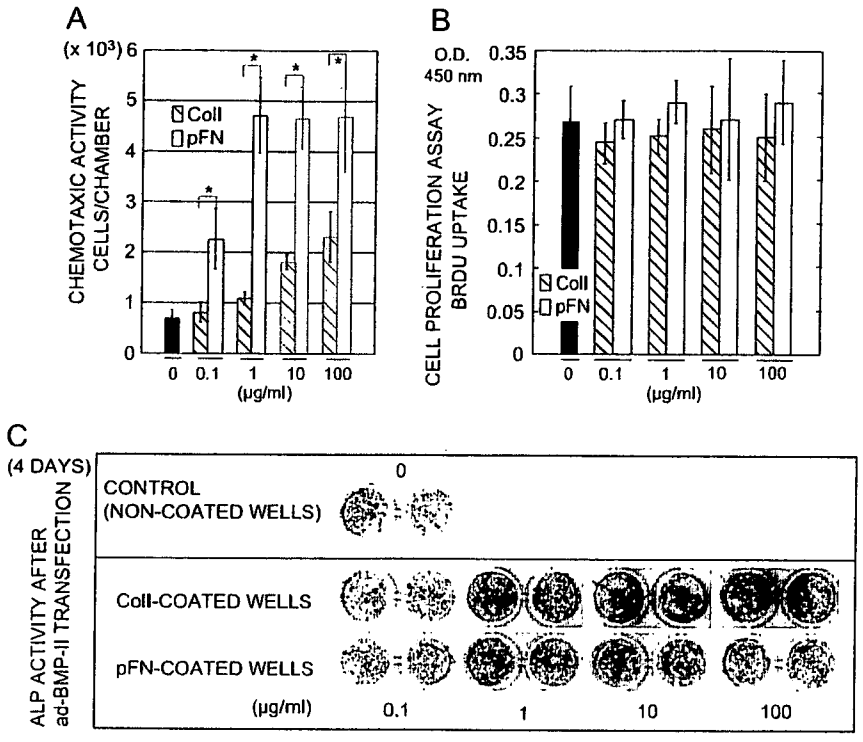

Fig. 6. (A) Chemotaxis assay after 24 h of culturing. The chemotactic activity was significantly higher in the pFN-coated group at all concentrations tested. (B) Proliferation assay 24 h after culturing. The proliferation activity was quantified by the Biotrak™ cell proliferation ELISA system (OD 450 nm). No significant difference in proliferation activity was seen. The values represent means ± SD of three separate experiments performed in triplicate (*$p < 0.05$). (C) The effect of Coll or pFN on the induction of ALP activity in BMSCs. Four days after the transfection of the ad-BMSCs (MOI = 5), the cells cultured on each matrix were fixed and stained as described above with *p*-nitrophenyl phosphate as a substrate. Two representative results of at least three independent experiments are shown. No remarkable difference in differentiation was observed.

imply that pFN may be an important factor for the initial stages of osseointegration, which have a profound impact on the bone–implant stability. Hence, strategies to induce the adsorption of pFN onto the implant would accelerate osseointegration and substantially reduce the stability dip if a sufficient amount of pFN can be adsorbed. There are two possible strategies for the progressive adsorption of pFN onto the implant surface. One is to mechanically coat the implant with pFN, as done in this study. The other is to physico-chemically modify the implant surface in order to render it optimal for further accumulation of the endogenic pFN. Deligianni et al. [34] have stated that higher amounts of FN were found on rough titanium surfaces compared to smoother surfaces. The surface roughness of the model we used in this study was Ra = 0.815, a relatively smooth surface, and the reduction of the pFN signal could be seen by day 2 in the control group. It may well be useful to roughen the surface not only to ensure the longer duration of pFN around the implant but also to enable the mechanical coating of the implant surface with a larger amount of pFN.

The observed remarkable enhancement of new bone apposition on the pFN-coated implant surface during the initial stages of osseointegration is promising and could lead to a significant reduction of the healing period in clinical applications.

5. Conclusion

This current study using the newly developed titanium ion-plated acrylic implant experimental system showed that coating pFN onto the implant surface induced earlier osseointegration than that of the non-coated control. *In vitro* chemotaxis assay showed chemotaxis of cFN-positive BMSCs by the effect of pFN. The *in vivo* and *in vitro* results suggested that adsorption of pFN onto the implant surface was effective for earlier osseointegration due to the release of pFN and for the subsequent chemotaxis of cFN-positive BMSCs with an osteogenic potential.

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Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup

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Abstract

As part of the Fourth International Workshop on Genotoxicity Testing (IWGT), held 9–10 September 2005 in San Francisco, California, an expert working group on the Comet assay was convened to review and discuss some of the procedures and methods recommended in previous documents. Particular attention was directed at the in vivo rodent, alkaline (pH >13) version of the assay. The aim was to review those protocol areas which were unclear or which required more detail in order to produce a standardized protocol with maximum acceptability by international regulatory agencies. The areas covered were: number of dose levels required, cell isolation techniques, measures of cytotoxicity, scoring of comets (i.e., manually or by image analysis), and the need for historical negative/positive control data. It was decided that a single limit dose was not sufficient although the required number of dose levels was not stipulated. The method of isolating cells was thought not to have a qualitative effect on the assay but more data were needed before a conclusion could be drawn. Concurrent measures of cytotoxicity were required with histopathological examination of tissues for necrosis or apoptosis as the "Gold Standard". As for analysing the comets, the consensus was that image analysis was preferred

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but not required. Finally, the minimal number of studies required to generate a historical positive or negative control database was not defined; rather the emphasis was placed on demonstrating the stability of the negative/positive control data. It was also agreed that a minimum reporting standard would be developed which would be consistent with OECD in vivo genotoxicity test method guidelines.

Keywords: Single cell gel assay; Comet assay; DNA damage; Genotoxicity; Alkaline electrophoresis

1. Introduction

The Comet assay, also referred to as the single cell gel electrophoresis (SCG or SCGE) assay, is a rapid, visual, and quantitative technique for measuring DNA damage in eukaryote cells [1–7]. Under alkaline (pH >13) conditions, the assay can detect single and double-stranded breaks, incomplete repair sites, alkali labile sites, and also possibly both DNA–protein and DNA–DNA cross-links, in virtually any eukaryotic cell population that can be obtained as a single cell suspension.

As the Comet assay has gained in popularity as a standard laboratory technique for evaluating DNA damage and/or repair, the question of how it can be applied within the current regulatory strategy of genotoxicity testing has become a matter of debate [8]. The primary focus of interest has been on the alkaline (pH >13) version, as it is applied to *in vivo* genotoxicity testing strategies [6,8–11]. This is especially important now that acceptance of the *in vivo* Comet assay by regulatory agencies in a number of countries is growing, with some already citing it as an acceptable second test [12,13]. Part of the reason for this acceptance has been the development of a standard protocol and acceptance criteria for the assay through the IWGT working parties [6] and international Comet assay workshops [10]. The purpose of this meeting was to review the procedures and methods recommended in previous guidance documents [6,10], with particular attention being given to the *in vivo* rodent alkaline (pH >13) assay.

Prior to the actual IWGT session, the members of the working group were assigned to different subgroups with each subgroup responsible for reviewing a particular topic. At the IWGT meeting, the subgroups presented their conclusions and recommendations to the complete working group for consideration and discussion, with input from the audience. This report provides an overview of the topics discussed and the consensus reached by the working group with regard to the *in vivo* rodent alkaline (pH >13) Comet assay (hereafter designated as the *in vivo* Comet assay).

2. Discussion topics and recommendations

2.1. Multiple dose levels versus limit dose

For this topic, the discussions focused on the number of dose levels to be used in the *in vivo* Comet assay, especially for cases where there is no evidence of animal toxicity. For example, as stated in the Organisation for Economic Development and Co-operation (OECD) test guideline 474 (rodent bone marrow micronucleus test), a chemical which shows no sign of toxicity up to the limit dose of 2 g/kg need only be tested at that dose [14].

The consensus of the working group was that a single dose level was not sufficient even for substances that could be tested at the limit dose of 2 g/kg. The reasoning behind this consensus was that there were not yet sufficient data to conclude that downturns in the dose response curve (i.e., a bell shaped dose response curve) would not occur for some substances due, for example, to altered bioavailability at higher dose levels. The 'downturn phenomenon', was also a matter of discussion among the members of the IWGT *in vivo* micronucleus (MN) group [15], where this phenomenon has been shown to occur in some MN studies although the underlying mechanism(s) have yet not been identified. In such cases, positive responses occurred at the second highest dose level. Therefore, it was concluded that the use of a single dose level could lead to problems in data interpretation. There was also the feeling that positive responses at multiple dose levels could reinforce the biological relevance of the result.

2.2. Cell isolation process

The background behind this discussion point was the disparate *in vivo* rodent Comet assay data sets published about *ortho*-phenyl phenol. When tested by Sasaki et al. [16], *ortho*-phenyl phenol was positive in the mouse using stomach, liver, kidneys, lung, urinary bladder as target organs. However, when tested by Bomhard et al. [17] in the same species, *ortho*-phenyl phenol was negative in the tissues investigated. One possible explanation for the difference in results was how the tissues were pro-

cessed. In Sasaki et al. [16], isolated nuclei were used, whereas in Bomhard et al. [17], isolated whole cells were used. Although there was much discussion on this subject along with data from two groups which showed that the method of tissue processing (i.e., isolated cells versus isolated nuclei) did not have a qualitative effect on the comet response, it was decided that more data were needed before a conclusion could be made and that any international validation study should consider both processing methods.

2.3. Concurrent measures of cytotoxicity

Cell death is a process that leads to DNA degradation. Thus, all test methods that evaluate primary DNA damage, including the Comet assay, have the potential to detect agents that are cytotoxic rather than genotoxic. However, since DNA damage in the Comet assay is assessed at the level of the individual cell, it is possible in some cases to identify dead or dying cells by their specific image. Under alkaline conditions, necrotic or apoptotic cells can result in comets with small or non-existent head and large diffuse tails [18] as observed in *in vitro* studies following treatment with cytotoxic, non-genotoxic compounds [19–21]. However, such images may not be uniquely diagnostic for apoptosis or necrosis since they may also be detected after treatment with high doses of radiation or high concentrations of strong mutagens [22]. For the *in vivo* Comet assay, only limited data are available to establish whether cytotoxicity results in increased DNA migration in tissues of experimental animals. It was discussed that migration levels detected at the time of sampling are dependent on the tissue and the slope of the dose response for a particular tested compound. For some chemicals, despite the presence of necrosis or apoptosis in target organs such as kidneys [23], testes [24], and liver or duodenum [11], an increase in DNA migration was not observed. In contrast, enhanced DNA migration was seen in cells isolated from the livers of mice dosed with carbon tetrachloride under conditions that also resulted in necrosis, as determined from a histopathological examination [25]. It is also possible that at cytotoxic doses, a decrease in DNA migration may be detected due to the loss of heavily damaged or dying cells during sample processing and/or electrophoresis.

There was consensus on the need to include measures of cytotoxicity and to address the possible effects of cytotoxicity in comet data interpretation. The suggested methods included: a dye exclusion test for membrane integrity and metabolic competency [26] and determining the frequency of cells with low molecular weight DNA using the neutral diffusion assay [6,27]. The "Gold Standard" for assessing levels of necrosis and apoptosis when an *in vivo* Comet assay gave positive results was concluded to be histopathology. It was pointed out that there was a need to standardize ways to present histopathological findings.

2.4. Image analysis (IA) or manual scoring

A variety of commercial and freeware IA systems are available for assessing DNA migration in individual cells. In addition, manual scoring can also be used to determine the length of DNA migration, the percentage of cells with and without migration, or the proportion of comets that can be "binned" into various migration categories (generally one of five, from undamaged to maximally damaged depending upon the tail length) [28]. However, a limitation of this categorization method may be a potential inability to take into account the density or shape of tails which can include short but dense tails and long but sparse tails depending on the effects of compounds tested. With IA systems, the most common parameters analyzed are the percentage DNA in the tail (% tail DNA), tail moment, and tail length and/or image length (referring to nucleus plus migrated DNA). The percentage DNA in the tail is generally defined as the fraction of DNA in the tail divided by the amount of DNA in the cell multiplied by 100, while the tail length is the distance from the middle or the estimated perimeter of the comet head to the last visible signal in the tail. There are several measures of tail moment. The one most commonly used, called the Olive tail moment, is the product of the amount of DNA in the tail and the mean distance of migration in the tail [29]. It is important to note that some parameters (e.g., tail moment) may be calculated differently among IA systems and this can lead to quantitative differences, which can be problematic when comparing inter-laboratory data.

The consensus was that IA is preferred but not required. Heavily damaged cells exhibiting a specific microscopic image (commonly referred to as hedgehogs) consisting of small or non-existent head and large and diffuse tails [18] potentially represent dead or dying cells and may be excluded from data collection. However, determining their frequency may be useful for data interpretation. If IA is used, then % tail DNA appeared to be the most linearly related to dose and the easiest to intuitively understand [30]. However, there was no consensus that this IA measure of DNA migration must be the only one used. If some measure of tail moment is used, than % tail DNA and tail length data must be provided also. Data on the distribution of migration among

cells should also be presented. This is accomplished by sorting cells within "bins" based on the metric used to evaluate DNA migration and presenting the data as the percentage of cells within each bin.

2.5. Historical negative/positive control data

The minimal number of studies needed was not defined but enough studies need to be conducted to demonstrate the stability of the negative/positive control data. Criteria for determining the acceptability of new studies, based on historical control data, should be developed for each tissue by each laboratory. There was discussion on the background responses for negative controls and there was a consensus that negative controls should exhibit measurable DNA migration. However, there was no consensus as to how much mean DNA migration was needed among the control cells. It was recognized that the ability to detect chemicals that predominantly induce DNA cross-linking, damage that reduces the ability of the DNA to migrate, depends on the extent of average DNA migration in the control cells. Investigators who are attempting to detect such chemicals will need to demonstrate the adequacy of their *in vivo* Comet assay protocol for this purpose.

2.6. Minimal reporting standards

It was agreed that to ensure that all studies can be independently evaluated, a minimum reporting standard for regulatory submissions and publications will be developed. This standard will be consistent with OECD *in vivo* genetic toxicology test method guidelines. Previous publications have covered some aspects of protocol design and reporting [10,31].

2.7. Conclusions

In recent years, the *in vivo* Comet assay has become increasingly used for regulatory purposes and acceptance of the test method by regulatory agencies is growing (reviewed in [8]). However, several issues on study design and on data analysis and assessment that required further investigation remain and it was these issues that were discussed by the IWGT working group. In addition to guidance provided in previous published guidelines [6,10], consensus among the participants of the working group was reached with regards to the selection of the number of dose levels, the need to include concurrent measures of cytotoxicity in the studies, the adequacy of manual scoring, and the need to develop historical control data. Consensus was also reached on the need for an international validation study to stringently evaluate the reliability and accuracy of the *in vivo* Comet assay (as well as *in vitro* versions). This validation study would compare, among other protocol issues, test results obtained using isolated nuclei versus isolated whole cells from various tissues.

Acknowledgements

During the preparation of this report, the Japanese Center for the Validation of Alternative Methods (JaCVAM) announced that they were forming a study management team including participants from the European Centre for the Validation of Alternative Methods (ECVAM), the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), US National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Mammalian Mutagenicity Study Group/Japanese Environmental Mutagen Society (MMS/JEMS) to conduct an international Comet assay validation study. This validation study is scheduled to start in late 2006 and will focus initially on the *in vivo* Comet assay, to be followed by the validation of various *in vitro* Comet assays. ECVAM has also implemented an initiative to evaluate the validity of the *in vitro* Comet assay.

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Mouse lymphoma thymidine kinase gene mutation assay: Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment

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Abstract

The Mouse Lymphoma Assay (MLA) Workgroup of the International Workshop on Genotoxicity Testing (IWGT), comprised of experts from Japan, Europe and the United States, met on September 9, 2005, in San Francisco, CA, USA. This meeting of the MLA Workgroup was devoted to reaching a consensus on issues involved with 24-h treatment. Recommendations were made concerning the acceptable values for the negative/solvent control (mutant frequency, cloning efficiency and suspension growth) and the criteria

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to define an acceptable positive control response. Consensus was also reached concerning the use of the global evaluation factor (GEF) and appropriate statistical trend analysis to define positive and negative responses for the 24-h treatment. The Workgroup agreed to continue their support of the International Committee on Harmonization (ICH) recommendation that the MLA assay should include a 24-h treatment (without S-9) in those situations where the short treatment (3–4 h) gives negative results.

Keywords: Mouse lymphoma assay; In vitro mutation; Thymidine kinase

1. Introduction

The Mouse Lymphoma Assay (MLA) Workgroup of the International Workshop on Genotoxicity Testing (IWGT), comprised of experts from Japan, Europe and the United States, met on September 9, 2005, in San Francisco, CA, USA. This meeting of the MLA Workgroup was devoted to reaching a consensus on issues related to the use of 24-h treatment.

The first meeting of the MLA Workgroup was held as a part of the International Workshop on Genotoxicity Testing Procedures in Washington, DC, in the spring of 1999. Since that time, the Workgroup has been working to address three main issues of importance to the assay. These include: (1) the conduct of a data-based analysis and a final recommendation for using the relative total growth (RTG) as the appropriate measure for cytotoxicity; (2) the criteria for data acceptance (based on the negative/vehicle and positive controls) and a new method [the global evaluation factor (GEF)] for data evaluation; (3) the issues related to the International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) recommended use of a 24-h treatment time (including the ability of the assay to detect aneugens). This is the 5th meeting of the Workgroup in which consensus has been reached and reported. The previous four meetings are reported in Moore et al. [1–4].

2. 24-h treatment

Following the ICH recommendation requiring that the MLA be conducted using a 24-h treatment (without S-9) in situations where the short treatment (3–4 h) was negative, laboratories have conducted such experiments. With the goal of determining the approximate frequency at which chemicals require a 24-h treatment to express their mutagenic potential, and to make recommendations concerning the conduct of the 24-h treatment, the Workgroup solicited data from laboratories conducting both the agar and microwell versions of the assay.

Laboratories were asked to evaluate data obtained since 2002 (some earlier data were submitted) and to base a positive result on the assay evaluation criteria in force for each participating laboratory at the time of the performance of the assay. They were asked to identify: (i) compounds uniquely positive following 24-h treatment in the absence of S-9, (ii) compounds positive following short (3- or 4-h) treatment times in the absence of S-9, but negative following 24-h treatment in the absence of S-9, and (iii) compounds either known or suspected to be aneugens.

An estimated 990 data sets (compounds) were reviewed by the individual laboratories to identify compounds fitting into one of these three categories. The majority of assays (approximately 900) were performed using the microwell method; approximately 90 assays used the agar method. Of these approximately 990 tests, 71 (7%) were positive, as assessed by the individual laboratory. It should be noted that the nine laboratories that submitted data only provided the actual data for these 71 test agents. These data were compiled, analyzed and summarized by three members of the Workgroup and the summary information was used in the deliberations of the entire Workgroup. It should be noted that only five data sets were submitted for the third category (known or suspected aneugens). This small number was considered insufficient to permit meaningful analysis and although the data was compiled, there was no discussion of this category by the Workgroup.

2.1. Category 1 responses

A total of 56 data sets (54 in the microwell assay and 2 in the agar assay) were uniquely positive following 24-h treatment in the absence of S-9. The negative controls for each of the 56 data sets were evaluated to determine whether they met all of the revised assay acceptance criteria agreed in the 3rd Workgroup meeting, held in Plymouth in 2002 [3] and the acceptable range for solvent control mutant frequencies (MF) agreed in the 4th Workgroup meeting held in Aberdeen in 2003 [4]. The application of these acceptance criteria eliminated 19 data sets. See Fig. 1 for a breakdown as to the causes of the unacceptable experiments.

A number of the remaining 56 data sets showed only a very small induced MF (IMF). In fact, the maximum

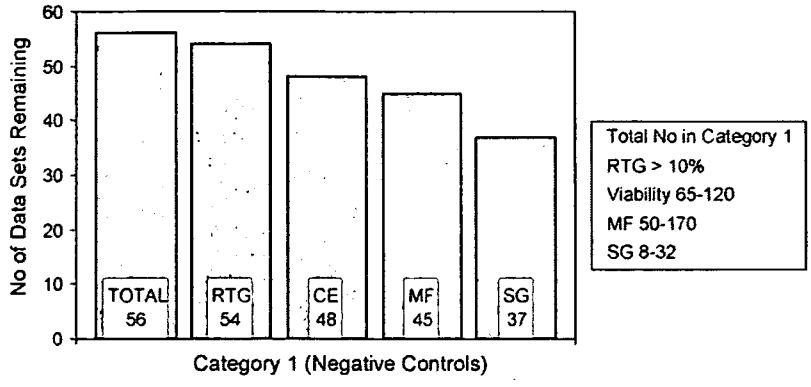

Fig. 1. Column graph demonstrating how many Category 1 (unique 24-h positive) data sets would be excluded as the acceptance criteria [4] are applied sequentially left to right. Note that SG as described in this instance were the values recommended for the short term treatment. They were applied to the 2 day expression period and excluded the 24-h treatment.

increase in MF observed at any data point (compared to concurrent controls) did not exceed the GEF in 26 out of 56 data sets (Fig. 2). Therefore, these data sets did not meet the new criteria required for a positive response, as agreed in the 4th Workgroup meeting in Aberdeen in 2003 [4]. Positive responses are defined as those that exceed the GEF and show statistically positive dose response trends (see discussion below concerning data evaluation).

After applying both the acceptance criteria for the negative controls and the GEF, the number of unique 24-h positive compounds was reduced to 18.

2.2. *Category 2 responses*

Only 10 data sets (9 in the microwell assay and 1 in the agar assay) were positive following the short (3- or 4-h) treatment in the absence of S-9, but apparently negative following 24-h treatment in the absence of S-9. Application of the acceptance criteria to the negative controls in these data sets reduced this number to 7 and the additional application of the GEF to the data further reduced the number to 4 (Fig. 3). The Workgroup made no additional recommendations concerning the 3–4 h treatment, based on this data.

2.3. *Acceptance criteria for negative/vehicle controls (24-h treatment)*

Previously, following an extensive evaluation of negative/vehicle control data from a number of laboratories using the short (3- or 4-h) treatment time, the Workgroup reached consensus on the acceptance criteria for individual experiments based upon several negative/vehicle control parameters [4]. With the exception of the suspension growth (SG) parameter, the Workgroup recommended that the same criteria be applied to the 24-h treatment experiments. Because the 24-h treatment includes 3 rather than 2 days of suspension growth, the acceptance criteria for the 24-h SG was revised to 32–180. The theoretical optimum suspension growth is about 5-fold per day, or 125 over the 3-day

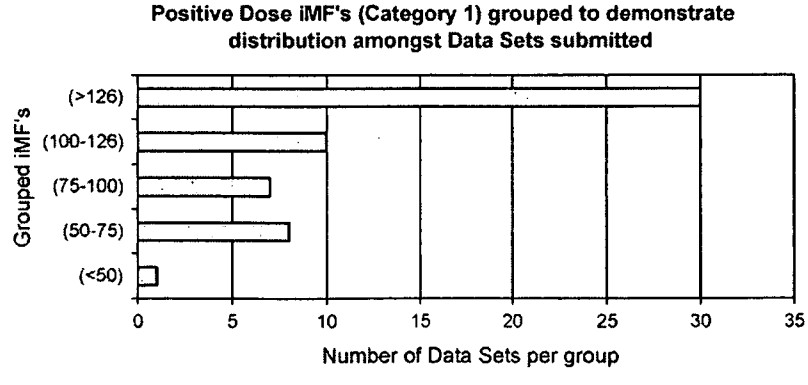

Fig. 2. Bar chart demonstrating breakdown of the 56 Category 1 (unique 24-h positive) studies in terms of the induced MF of highest positive data point.

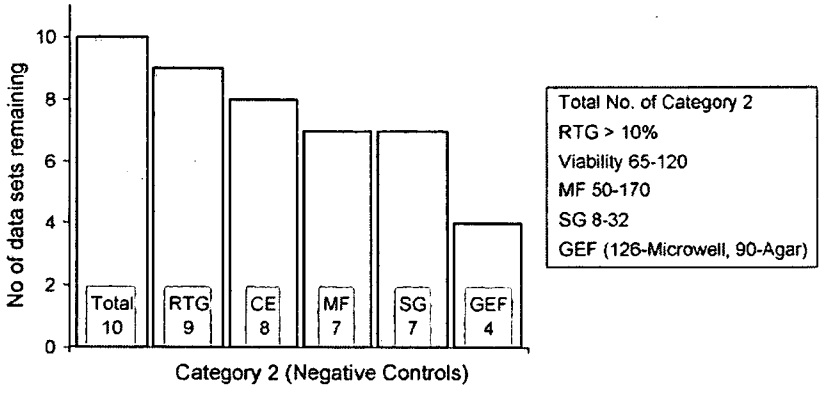

Fig. 3. Column graph demonstrating the number of Category 2 (unique 3- or 4-h positive) data sets that would be excluded as stated acceptance criteria [4] are applied in sequence of left to right.

period. However, there is variability in growth rates and the Workgroup would be very concerned with suspension growth less than 32. The high limit of 180 allows for reasonable errors in cell counting and dilution. As with the short treatment, the acceptance criteria for the background MF are specific to the agar or microwell version of the assay. For both methods the cloning efficiency (CE) referred to in the criteria is the absolute CE obtained at the time of mutant selection. The suspension growth of the negative/vehicle control refers to the growth during both the 24-h treatment and the 2-day expression period following treatment. It is defined as the fold-increase of the cell number during this 3-day period. The SG is calculated by the treatment period fold-increase multiplied by both the expression day 1 and expression day 2 fold-increases in cell number.

The acceptance criteria for the negative/vehicle control parameters for the soft agar and microwell methods of performing the MLA using 24-h treatment are now as follows:

Agar method	Microwell method
MF: 35–140 × 10^{-6}	MF: 50–170 × 10^{-6}
CE: 65–120%	CE: 65–120%
SG: 32–180	SG: 32–180

2.4. Acceptance criteria for positive controls (24-h treatment)

As with the short treatment time experiments, positive control cultures should be included in every 24-h treatment MLA experiment. To assess the adequacy of detection of both small and large colony mutants in the 24-h treatment, the Workgroup agreed that it is appropriate to apply the same acceptance criteria developed for the short treatment time. There are two equally acceptable approaches to assuring an adequate positive control response. (1) The laboratory should use a dose of a mutagenic chemical that yields an absolute increase in total MF that is an increase above the spontaneous background MF [an induced MF] of at least 300 × 10^{-6}. The small colony MF should account for at least 40% of that IMF. For instance, in a culture showing an IMF of 300 × 10^{-6}, the small colony IMF should be at least 120 × 10^{-6}. (2) The second approach requires the use of a dose of a chemical that increases the small colony MF at least 150 × 10^{-6} above that seen in the concurrent negative/vehicle control (a small colony IMF of 150 × 10^{-6}).

In addition, the upper limit of cytotoxicity observed in the positive control culture should have a relative total growth (RTG) that is greater than 10% [2]. The Workgroup recognizes that some laboratories prefer to use more than one dose of their positive control and/or to use a dose that gives a small increase in MF. For these laboratories, it is sufficient if only a single dose of the positive control meets the acceptance criteria.

2.5. Data evaluation

Once the criteria for experimental acceptance have been satisfied, the data from each individual experiment can be evaluated to determine whether the response is positive, negative or equivocal. The Workgroup agreed that data generated using 24-h treatment should be evaluated using the same method previously developed by the Workgroup for use with the short treatment times. A brief summary of the previous analyses conducted by the Workgroup and the rationale for developing the new method using the GEF in conjunction with appropriate statistical analysis to ascertain the presence of a dose-related positive trend is included in the Plymouth and Aberdeen Meeting Reports [3,4]. It should

be noted that the GEF approach takes into account previous guidance documents (i.e. FDA Redbook [http://www.cfsan.fda.gov/~redbook/red-toca.html] and OECD [5], which states that biological relevance should be a major factor in data evaluation.

The GEF evaluation method requires that the IMF exceeds a value based on the global distribution of the background MF for each method (agar or microwell). This value, the GEF, was established by the Workgroup, based on short treatment experiments, to be 126 for the microwell version of the assay and 90 for the agar version. The GEF is applied as follows: if the negative/vehicle control MF in a microwell experiment is 100×10^{-6}, then one of the treatment groups must have a MF of at least $100 + 126$ (the microwell GEF) $= 226 \times 10^{-6}$ in order to meet the GEF criterion for a positive call. An appropriate statistical trend test should be applied to determine whether there was a positive dose-related increase.

A test agent response in an experiment is positive if both the IMF for any treatment meets or exceeds the GEF and a positive trend test is obtained. A test agent response is clearly negative if both the trend analysis and the GEF are negative. Situations where either (but not both) the GEF or statistical analysis is positive should be evaluated on a case-by-case basis. It should be noted that it is generally advisable to conduct one or more additional experiments to better define the assay response (particularly in the 30–10% RTG cytotoxicity range).

For more detail on the Workgroup recommendations on the steps for proper assay evaluation, the reader is referred to the summaries of the New Orleans, Plymouth and Aberdeen meetings [2–4]. All of these recommendations are equally applicable to the short treatment and the 24-h treatment.

3. Conclusions

From this analysis, it is clear that only a very small percentage of chemicals (less than 2%) are uniquely positive at 24 h, and an even smaller percentage appear to be uniquely positive at short (3- or 4-h) treatment times. The low numbers of unique 3- or 4-h positive results may be attributable to the regulatory guideline requirements that positive results observed following 3- or 4-h treatments do not need to be further evaluated at 24 h. In some of the unique 24-h treatment cases, the longer treatment time provides for the effective treatment of a higher dose of compound. This is particularly true when a chemical's insolubility prevents testing to adequate toxicity in the short treatment time. There are also some situations in which the maximum recommended concentration (5000 μg/ml) was not mutagenic and was insufficiently toxic in the short treatment, but was mutagenic following a 24-h treatment. There is also evidence that some (but not all) aneugens require longer treatment time [6].

Based upon all the available data, the Workgroup agreed to continue its support of the ICH recommendation that 24-h treatment be used when the short treatment time is negative or equivocal.

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Potassium bromate treatment predominantly causes large deletions, but not GC > TA transversion in human cells

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Abstract

Potassium bromate ($KBrO_3$) is strongly carcinogenic in rodents and mutagenic in bacteria and mammalian cells in vitro. The proposed genotoxic mechanism for $KBrO_3$ is oxidative DNA damage. $KBrO_3$ can generate high yields of 8-hydroxydeoxyguanosine (8OHdG) DNA adducts, which cause GC > TA transversions in cell-free systems. In this study, we investigated the in vitro genotoxicity of $KBrO_3$ in human lymphoblastoid TK6 cells using the comet (COM) assay, the micronucleus (MN) test, and the thymidine kinase (*TK*) gene mutation assay. After a 4 h treatment, the alkaline and neutral COM assay demonstrated that $KBrO_3$ directly yielded DNA damages including DNA double strand breaks (DSBs). $KBrO_3$ also induced MN and *TK* mutations concentration-dependently. At the highest concentration (5 mM), $KBrO_3$ induced MN and *TK* mutation frequencies that were over 30 times the background level. Molecular analysis revealed that 90% of the induced mutations were large deletions that involved loss of heterozygosity (LOH) at the *TK* locus. Ionizing-irradiation exhibited similar mutational spectrum in our system. These results indicate that the major genotoxicity of $KBrO_3$ may be due to DSBs that lead to large deletions rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed. To better understand the genotoxic mechanism of $KBrO_3$, we analyzed gene expression profiles of TK6 cells using Affymetrix Genechip. Some genes involved in stress, apoptosis, and DNA repair were up-regulated by the treatment of $KBrO_3$. However, we could not observe the similarity of gene expression profile in the treatment of $KBrO_3$ to ionizing-irradiation as well as oxidative damage inducers.

Keywords: Potassium bromate ($KBrO_3$); *TK*-mutation; Loss of heterozygosity (LOH); 8-Hydroxydeoxyguanosine (8OHdG); Gene expression profile

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1. Introduction

Potassium bromate ($KBrO_3$) is used as in bread making a flour improver and in the production of fish-pastes. The EU countries now prohibit its use as a food additive because of its carcinogenicity. Japan and the USA, however, permit its use in bread making on the condition that it never remains in the final product. $KBrO_3$ causes tumors, especially in kidney, in rats, and mice after long-term oral administration in drinking water [1–3]. $KBrO_3$ is also genotoxic. It is positive in in vitro genotoxicity tests – including the bacterial reverse mutation assay [1], the chromosomal aberration test conducted in Chinese hamster cells [4], and the mouse lymphoma assay [5] – and in vivo in the micronucleus test (MN) [6,7].

It has been proposed that $KBrO_3$ induces tumors through the production of oxidative damage to DNA. Oxidative DNA damage can cause mutations that contribute to the activation of oncogenes and/or the inactivation of tumor suppressor genes, thereby leading to tumorigenesis [8,9]. 8-Hydroxydeoxyguanosine (8OHdG) is the main form of oxidative DNA damage induced by $KBrO_3$ [10]. It primarily causes GC > TA transversions (as a result of the pairing of 8OHdG with A) and is believed to be responsible for mutagenesis, carcinogenesis, and aging [11,12]. $KBrO_3$ increases 8OHdG DNA adducts in vivo and in vitro [13–15]. However, $KBrO_3$ induces mutations weakly in microbial mutation assays and the *Hprt* mutation assay in mammalian cells, while it induces chromosome aberrations strongly both in vivo and in vitro [1,16,17]. These findings raise the question of whether 8OHdG is required for the mutagenic process involved in $KBrO_3$-induced carcinogenesis.

In the present study, we examined the genotoxic properties of $KBrO_3$ using the comet assay (COM), the MN test, and thymidine kinase (*TK*) gene mutation assays in human lymphoblastoid TK6 cells [18]. Unlike the X-linked hemizygous *HPRT* gene mutation assay, the *TK* mutation assay can detect not only point mutations, but also large scale chromosomal deletions, recombinations, and aneuploidy [19–21]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumed relevant to carcinogenesis. We analyzed the *TK* mutants induced by $KBrO_3$ at the molecular level and investigated what kind of mutation predominated. We also profiled global gene expression in TK6 cell exposed to $KBrO_3$ using Affymetrix GeneChip® Expression analysis to understand the genotoxic mechanism of $KBrO_3$.

2. Materials and methods

2.1. Cell culture, chemicals, and treatment

The TK6 human lymphoblastoid cell line has been described previously [22]. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Life Technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JR Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere with 100% humidity. $KBrO_3$ (CAS No.7758-01-2) was purchased from Wako Pure Chemical Co. (Tokyo) and dissolved in RPMI medium just before use.

We prepared 20 ml aliquots of cell suspension at a concentration of 5.0×10^5 cells/ml in 50 ml polystyrene tubes. Different concentrations of $KBrO_3$ were added to the tubes, which were then placed on a platform shaker and incubated at 37 °C for 4 h with gentle shaking. At the end of the treatment period, the cell cultures were centrifuged, washed once, and re-suspended in fresh medium. We cultured them in new flasks for the MN assay and *TK* gene mutation assay, or diluted them for plating for survival estimates.

2.2. Genotoxicity assays

After treating cells with $KBrO_3$, we prepared slides for conducting the alkaline and neutral COM assay. The alkaline COM assay was performed as previously reported [23]. For the neutral COM assay, the slide was electrophoresed with chilled neutral solution (pH 8) containing of 90 mM Tris, 2 mM Na_2EDTA, and 90 mM boric acid according to the method by Wada et al. [24]. The COM slides were stained with SYBER green (Molecular Probes, Eugene, OR) and observed by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and tail length of the comet was measured. The relationship between $KBrO_3$ treatment and migration was statistically analyzed by the Dunnett test [25].

We prepared the MN test samples 48 h after treatment, as previously reported [23]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold methanol containing 25% acetic acid, then re-suspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 μg/ml acridine orange solution and immediately observed with the aid of an Olympus model BX50 fluorescence microscope equipped with a U-MWBV band pass filter. At least 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test [26].

We prepared the *TK* gene mutation assay samples 3 days after treatment. We seeded cells from each culture into 96-well plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluo-

rothymidine (TFT). We also plated 1.6 cells/well without TFT to determine plating efficiency. All plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 14 days, we scored colonies on the PE plates and the normal-growing (NG) *TK* mutants on the TFT plates, then re-fed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing (SG) *TK* mutants. Mutation frequencies, relative survival (RS), and relative suspension growth (RSG) were calculated as previously described [23]. The data of mutant frequencies were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [27].

2.3. LOH analysis of TK mutations by polymerase chain reaction (PCR)

To avoid analyzing identical mutants, we performed an additional *TK* mutation assay and isolated *TK* mutants from independent culture after a 4 h treatment with 2.5 mM $KBrO_3$. We confirmed the phenotype of the *TK* mutant clones by re-challenging them with TFT medium. We also determined the growth rate of the clones and confirmed whether they were NG or SG mutants.

Genomic DNA was extracted from the *TK* mutant cells and used as a template for PCR. We conducted the PCR-based LOH analysis of the human *TK* gene as described previously [28]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that is heterozygous for frame shift mutations. A third primer set for amplifying parts of the β-globin was also used as the internal control. We applied quantitative-multiple PCR for co-amplification of the three regions. The PCR products were analyzed with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and were classified into "no LOH", "hemizygous (hemi-) LOH", or "homozygous (homo-) LOH". To determine the extent of the LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis [28]. The results were processed by GenoTyper™ software (PE Biosystems, Chiba, Japan) according to the manufacturer's guidelines.

2.4. Gene expression analysis

Total RNA was isolated from the TK6 cells after 4 h treatment with 2.5 mM $KBrO_3$ and was purified by RNeasy columns (Qiagen, Valencia, CA). We conducted a single cDNA synthesis, cRNA labeling, and cRNA fragmentation according to the manufacturer's recommendations (Affymetrix Inc., Santa Clara, CA) and employed Affymetrix GeneChip Expression analysis. The hybridization mixture for each sample was hybridized to an Affymetrix U133A human genome array. We processed the scanned data using Microarray Suite Software Version 5.0 (Affymetrix Inc., Santa Clara, CA) and imported the data into GeneSpring software (Silicon Genetics, Redwood City, CA). Signal intensity was normalized by per-gene and per-chip, and the ratios were calculated by normalizing $KBrO_3$ sample to the corresponding control sample. We used intensity-dependent (step-wise) selection of significant changes with higher cut-off value for lower signal intensity (1.75-, 2.0-, 2.25-, 2.5-, and 3.5-fold for genes intensity range of >1000, 500–1000, 100–500, 50–100, and 10–50, respectively), and up-regulated genes with a presence call in $KBrO_3$ sample, whereas down-regulated genes with a presence call in the control sample.

3. Results

3.1. Cytotoxicity and genotoxicity of $KBrO_3$

$KBrO_3$ exerted strong and concentration-dependent cytotoxicity in TK6 cells (Fig. 1). It induced approximately 50% cytotoxicity (51% RSG and 44% RS) at 2.5 mM. To investigate whether $KBrO_3$ directly causes DNA damage, we conducted the COM assay. Induction of COM tail after the treatment of in alkaline version was statistically significant 2.5 and 5 mM. In the neutral COM assay, the induction was observed form the lower concentration (Fig. 1). Because the neutral COM is thought to be associated with DNA double strand breaks (DSBs) [29], this result indicates that $KBrO_3$ directly causes DNA damage including DSBs. $KBrO_3$ also induced MN and *TK* mutation in a concentration-dependent manner and their inductions were statistically significant (Fig. 1). At the maximum concentration, it induced both MN and *TK* mutation frequencies about 30 times the control values. Two distinct phenotypic classes of *TK* mutants were generated: NG mutants grew at the same rate as the wild type (doubling time 13–17 h), and SG mutants grew at a slower rate (doubling time > 21 h). NG mutants result from intragenic mutations, while SG mutants result from gross changes (extending beyond the *TK* gene) [20]. $KBrO_3$ predominantly induced SG mutants (Fig. 1), implying that $KBrO_3$ treatment predominantly causes gross structural changes, but not small genetic alterations such as point mutations.

3.2. Molecular analysis of TK mutants

The *TK* mutants were randomly isolated from independent cultures treated with 2.5 mM $KBrO_3$ for 4 h. Table 1 shows the cytotoxicity (RSG), mutation frequency, and proportion of SG mutants induced by $KBrO_3$. We subjected 40 induced mutants to LOH analysis. Of those, 32 (80%) were SG mutants, which corresponded closely to the percentage of SG mutants induced in the assay (74.1%), indicating that the result of LOH analysis reflected the character of the induced

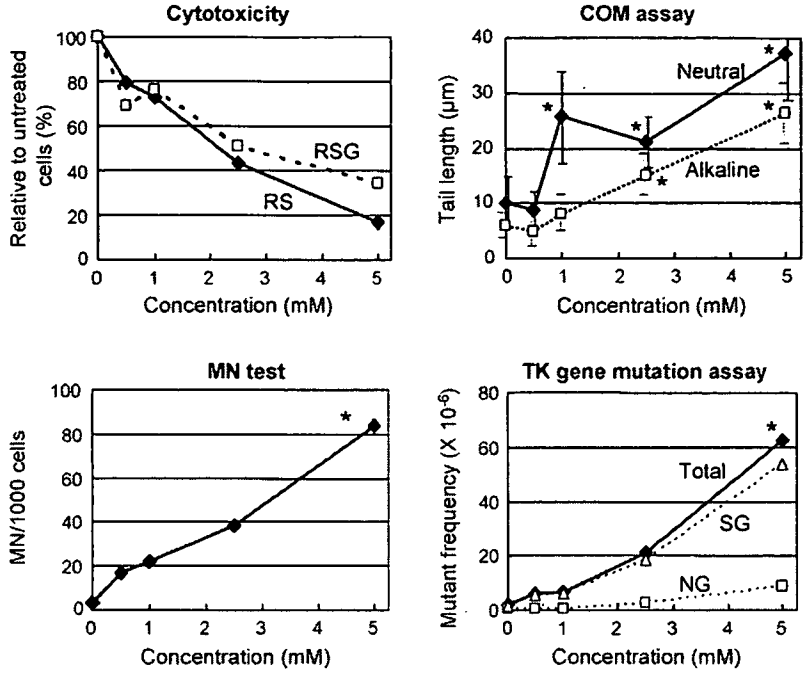

Fig. 1. Cytotoxic (relative survival, RS; relative suspension growth, RSG) and genotoxic responses (COM assay, MN test, and *TK* gene mutation assay) of TK6 cells treated with $KBrO_3$ for 4 h. Asterisk (*) statistically significant in Dunnett's test ($P < 0.05$) in COM assay, and in both pair-wise comparison and trend test ($P < 0.05$) in MN test and *TK* gene mutation assay.

mutations. Table 1 also shows the results of LOH analysis of the induced and spontaneously occurring mutants. The result of molecular analysis of spontaneous *TK* mutants was reported previously [21]. We classified the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH), and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [20]. Among the $KBrO_3$-induced mutants, 63% of NG mutants and 84% of SG mutants were hemi-LOH. In spontaneous mutants, on the other hand, majority of NG and SG mutants were non-LOH and homo-LOH, respectively. These results indicated that $KBrO_3$ predominantly induced large deletions. We previously reported the mutational spectra of *TK* mutants in TK6 cells that treated with the alkylating agent ethylmethane sulfonate (EMS), or X-irradiated [20,21]. Fig. 2 shows the comparison of the mutational spectra of spontaneous and induced *TK* mutants by EMS, X-irradiation, and $KBrO_3$. The mutation spectrum induced by $KBrO_3$ was similar to that induced by X-radiation (which also induces LOH, predominantly via deletion [21]) but not by EMS. The majority of the mutations induced by $KBrO_3$ were large deletions, but not point mutations.

Fig. 3 shows the regions of LOH and the distribution of spontaneous, X-ray-induced, and $KBrO_3$-induced

Table 1
Cytotoxic and mutational responses to $KBrO_3$, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) *TK* mutants

Treatment	Cytotoxic and mutational response			LOH analysis at *TK* gene (%)			
	RSG (%)	MF ($\times 10^{-6}$)	% SG	Number	Non-LOH	Hemi-LOH	Homo-LOH
Spontaneous[a]	100	2.19	56	56			
NG mutants				19	14(74)	3(16)	2(11)
SG mutants				37	0(0)	9(24)	28(76)
$KBrO_3$ (2.5 mM)	51	29.4	74	39			
NG mutants				8	3(37)	5(63)	0(0)
SG mutants				31	1(3)	27(84)	4(13)

[a] Data from Zhan et al. [22].

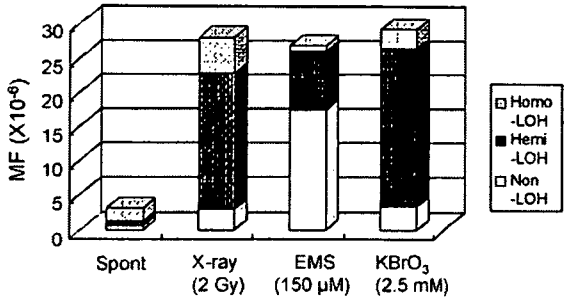

Fig. 2. *TK* mutation spectra in untreated, X-ray-treated (2 Gy), EMS-treated (150 μM, 4 h), and $KBrO_3$-treated (2.5 mM, 4 h) TK6 cells. The fraction of each mutational event was calculated by considering the ratio of NG to SG mutants and the results of molecular analysis (Table 1). The data for all but the $KBrO_3$ treatments were taken from our previous paper [20].

LOH mutants. $KBrO_3$ predominantly induced hemi-LOH, the result of large interstitial and terminal deletions, which we also frequently observed in the X-ray-induced LOH mutants. These results indicate that the genetic changes induced by $KBrO_3$ were similar to those induced by X-rays.

3.3. Gene expression analysis

Table 2 lists the genes that significantly increased expression following exposure to 2.5 mM $KBrO_3$. These genes are involved in stress response (6 genes), cell growth and DNA repair (19 genes), immune response (3 genes), apoptosis (3 genes), signal transduction (10 genes), transcription regulation (10 genes), chromosome organization (2 genes), protein modification (7 genes), energy metabolism (6 genes), lipid metabolism (2 genes), purine biosynthesis (3 genes), and unclassified functions (42 genes). Table 3 shows the genes whose expression was suppressed by the treatment. The number of up-regulated genes was greater that the number of down-regulated genes.

4. Discussion

$KBrO_3$ is a complete carcinogen, possessing both initiating and promoting activities in rodents [1]. While it shows clear positive responses in the COM assay, MN test, and chromosome aberration test using mammalian cells [4,14,17], the mutagenic potential of $KBrO_3$ in bacteria and the *Hprt* assay in Chinese hamster cells is weak or negative [1,14,17,30]. In our present study, $KBrO_3$ treatment strongly induced *TK* gene mutations. The reason we observed the induction of gene mutations and others did not is that $KBrO_3$ induces detectable mutagenecity in the *TK* gene but are only weakly mutagenic or non-mutagenic in the *Hprt* gene and in microbial assays [20]. The lower mutation frequency in the *Hprt* gene is due to the low recovery of large deletions, which are not detected because they are lethal. $KBrO_3$ is positive in mouse lymphoma cell assays that target the *Tk* gene [5]. In in vivo genotoxicity tests, $KBrO_3$ strongly induces MN in male ddY mice but is only weakly mutagenic in the *gpt* mutation assay in transgenic mice, which mainly detects point mutations and small deletions [31]. These results indicate that the property of genotoxicity

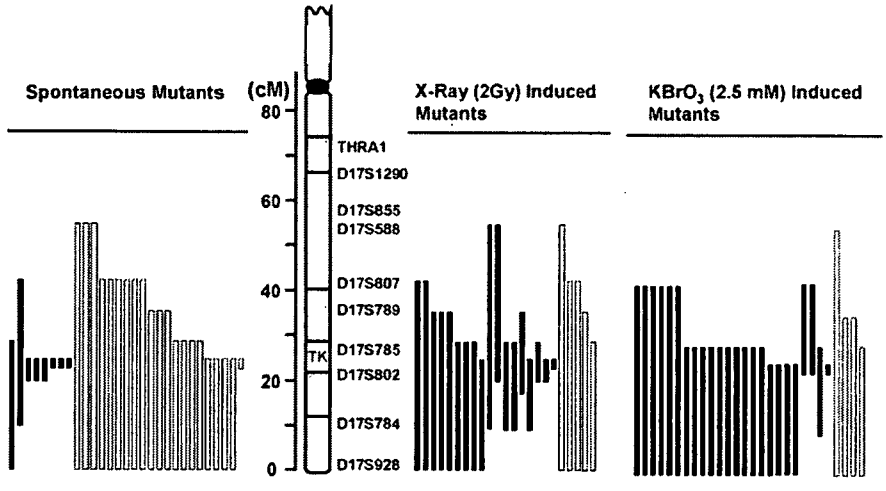

Fig. 3. The extent of LOH at the *TK* locus of TK6 cells that were untreated, X-ray-irradiated (2 Gy), or exposed to $KBrO_3$ (2.5 mM, 4 h). We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human *TK* locus maps to 17q23.2. Open and closed bars represent homozygous LOH and hemizygous LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 28 LOH mutants (4 NG and 24 SG). The data on spontaneous and X-ray-induced mutants were taken from our previous paper [20].

Table 2
Genes whose expression was up-regulated by $KBrO_3$ (2.5 mM, 4 h)

	Gene symbol	Ratio	Gene title
Stress response	CAT	2.77	Catalase
	DNAJC7	2.33	DnaJ (Hsp40) homolog, subfamily C, member 7
	FKBP5	2.87	FK506 binding protein 5
	HSPA8	3.02	Heat shock 70 kDa protein 8
	HSPCB	3.21	Heat shock 90 kDa protein 1, beta
	HSPD1	1.83	Heat shock 60 kDa protein 1
DNA repair, cell cycle, cell growth	BUB1	4.51	BUB1 budding uninhibited by benzimidazoles 1 homolog
	CCND2	5.08	Cyclin d2
	CCT2	3.33	Chaperonin containing TCP1, subunit 2 (beta)
	DKC1	2.37	Dyskeratosis congenita 1, dyskerin
	ENO1	2.10	Enolase 1 (alpha)
	HMGB1	2.16	High-mobility group box 1
	MAPRE1	2.32	Microtubule-associated protein, RP/EB family, member 1
	NME1	2.00	Non-metastatic cells 1, protein (NM23A) expressed in
	NOLC1	2.99	Nucleolar and coiled-body phosphoprotein 1
	NRAS	2.54	Neuroblastoma RAS viral (v-ras) oncogene homolog
	p21	3.22	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
	PPP2R1B	2.45	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform
	RAD21	2 34	RAD21 homolog
	RBBP4	2.00	Retinoblastoma binding protein 4
	RHOA	1.77	ras homolog gene family, member A
	SRPK1	2.75	SFRS protein kinase 1
	SSR1	2.66	Signal sequence receptor, alpha
Immune response	ARHGDIB	1.78	Rho GDP dissociation inhibitor (GDI) beta
	HLA-DRA	2.16	Major histocompatibility complex, class II, DR alpha
	IL2RG	2.43	Interleukin 2 receptor, gamma
Apoptosis	BCLAF1	6.42	BCL2-associated transcription factor 1
	FXR1	3.32	Fragile X mental retardation, autosomal homolog 1
	VDAC1	1.94	Voltage-dependent anion channel 1
Signal transduction	ANP32A	3.20	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
	OGT	2.74	O-linked *N*-acetylglucosamine (GlcNAc) transferase
	PIP5K1A	4.25	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha
	PLEK	2.95	Pleckstrin
	PTPN11	2.61	Protein tyrosine phosphatase, non-receptor type 11
	SPTLC1	2.62	Serine palmitoyltransferase, long chain base subunit 1
	SRPR	2.52	Signal recognition particle receptor
Transcription regulation	CDC5L	4.37	CDC5 cell division cycle 5-like
	HNRPC	4.40	Heterogeneous nuclear ribonucleoprotein C (C1/C2)
	MED6	2.45	Mediator of RNA polymerase II transcription, subunit 6 homolog
	MED6	2.45	Mediator of RNA polymerase II transcription, subunit 6 homolog
	NO NO	2.68	Non-POU domain containing, octamer-binding
	POLR1C	2.67	Polymerase (RNA) I polypeptide C, 30 kDa
	PRPF4	2.51	PRP4 pre-mRNA processing factor 4 homolog
Chromosome organization	CBX5	2.68	Chromobox homolog 5 (HP1 alpha homolog, Drosophila)
Protein modification	CANX	2.56	Calnexin
	COPA	6.55	Coatomer protein complex, subunit alpha
	EIF2S3	2.40	Eukaryotic translation initiation factor 2, subunit 3 gamma
	EIF4B	2.86	Eukaryotic translation initiation factor 4B
	RANBP2	3.96	RAN binding protein 2
	SEC23IP	2.67	SEC23 interacting protein

Table 2 (*Continued*)

	Gene symbol	Ratio	Gene title
Energy pathway	AFURS1	2.83	ATPase family homolog up-regulated in senescence cells
	CYB5-M	2.54	Cytochrome *b5* outer mitochondrial membrane precursor
	TOMM22	3.07	Translocase of outer mitochondrial membrane 22 homolog
Lipid metabolism	HMGCS1	2.58	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1
	SCD	2.56	Stearovl-CoA desaturase
Purine biosynthesis	ENTPD1	2.36	Ectonucleoside triphosphate diphosphohydrolase 1
	GART	2.64	Phosphoribosylglycinamide formyltransferase
	PAICS	1.79	Phosphoribosylaminoimidazole carboxylase
Unclassified	BANF1	2.77	Barrier to autointegration factor 1
	BAT1	1.95	HLA-B associated transcript 1///HLA-B associated transcript 1
	C1orf16	2.37	Chromosome 1 open reading frame 16
	CALU	2.40	Calumenin
	DAZAP2	2.57	DAZ associated protein 2
	DDX18	2.34	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18
	DHX9	9.37	DEAH (Asp-Glu-Ala-His) box polypeptide 9
	EXOSC2	3.03	Exosome component 2
	FLJ10534	2.07	Hypothetical protein FLJ10534
	FLJ10719	2.42	Hypothetical protein FLJ10719
	FLJ12973	2.76	Hypothetical protein FLJ12973
	GANAB	2.07	Glucosidase, alpha; neutral AB
	HEM1	2.37	Hematopoietic protein 1
	IGHM	2.76	Anti-HIV-1 gp120 V3 loop antibody DO142-10 light chain variable region
	IGKC	3.15	Anti-rabies virus immunoglobulin rearranged kappa chain V-region
	LIN7C	3.51	lin-7 homolog C (*C. elegans*)
	LOC54499	2.31	Putative membrane protein
	M6PR	3.59	Mannose-6-phosphate receptor
	MGC8902	2.27	Hypothetical protein MGC8902/
	MOBK1B	2.67	MOB1, Mps one binder kinase activator-like 1B (yeast)
	NS	2.15	Nucleostemin
	NUSAP1	3.25	Nucleolar and spindle associated protein 1
	OK/SW-cl.56	1.85	Beta 5-tubulin
	OPRS1	2.76	Opioid receptor, sigma 1
	PEG 10	2.50	Paternally expressed 10
	PEX19	2.34	Peroxisomal biogenesis factor 19
	PGK1	2.11	Phosphoglycerate kinase 1
	RPE	2.35	Ribulose-5-phosphate-3-epimerase
	SDBCAG84	3.16	Serologically defined breast cancer antigen 84
	SMU1	2.70	smu-1 suppressor of mec-8 and unc-52 homolog (*C. elegans*)
	TAGLN2	2.03	Transgelin 2
	UBC	2.65	Ubiquitin C
	XPNPEP1	2.84	X-prolyl aminopeptidase
	YWHAE	6.39	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
	YWHAZ	2.50	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

of $KBrO_3$ predominantly causes gross structural changes rather than small genetic changes such as point mutations.

$KBrO_3$ generates high yields of 8OHdG DNA adducts, which is a marker of oxidative DNA damage widely used as a predictor of carcinogenesis [10]. 8OHdG has been reported to be highly mutagenic in some experiments. In cell-free system, 8OHdG induced mutation by misincorporating adenine instead of cytosine [12]. Artificially incorporated 8OHdG at specific codons in a shuttle vector system efficiently induced GC > TA transversions in mammalian cells and *E. coli* [8,32,33]. In mammalian gene mutation assays in vitro and in vivo, however, the relationship between the accumulation of 8OHdG and the induction of GC > TA transversion has not been clear. Takeuchi et al.

Table 3
Genes whose expression was down-regulated by $KBrO_3$ (2.5 mM, 4 h)

	Gene symbol	Ratio	Gene title
Cell cycle, cell growth	FH	0.51	Fumarate hydratase
	MYC	0.55	v-myc myelocytomatosis viral oncogene homolog
Signal transduction	DUSP2	0.37	Dual specificityphosphatase 2
	RRBP1	0.39	Ribosome binding protein 1 homolog 180 kDa
	TBL3	0 43	Transducin (beta)-like3
Transcription regulation	CITED2	0.45	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
	KIAA1196	0.43	KIAA1196 protein
	TZFP	0 39	Testis zinc finger protein
Chromosome organization	H1FX	0.14	H1 histone family member X
Protein modification	CLTB	0.43	Clathrin, light polypeptide (Lcb)
Energy pathway	FDX1	0.45	Ferredoxin 1
	QPRT	0.41	Quinolinate phosphoribosyltransferase
	SLC39A4	0.43	Solute carrier family 39 (zinc transporter), member 4
Unclassified	BTBD2	0.35	BTB (POZ) domain containing 2
	LOC339229	0.44	Hypothetical protein LOC339229
	MGRN1	0.44	Mahogunin, ring finger 1
	MRP63	0.41	Mitochondrial ribosomal protein 63
	PHLDA1	0.43	Pleckstrin homology-like domain, family A, member 1
	PTPLA	0.37	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a
	SPATA2	0.45	Spermatogenesis associated 2

examined the mutagenicity of a hydroxyl radical generator, *N,N'*-bis (2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic diimide (NP-III). Although NP-III highly produced 8OHdG upon irradiation with UV in V79 cells, the frequency of *Hprt* gene mutation was not significantly induced [34]. Molecular analysis demonstrated the no association of induction of 8OHdG with GC > TA transversion in the *Hprt* mutants [35]. 8OHdG is mainly removed by Ogg1 protein in a manor of the base excision repair (BER) pathway. Arai et al. investigated the relationship between the accumulation of oxidative DNA damage and the induction of gene mutation using *Ogg1* deficient transgenic mice [36]. Although the 8OHdG level in kidneys of the *Ogg1* deficient mice increase 200 times of the control level after 4 weeks' $KBrO_3$ treatment, the mutation frequency in the transgenic *gpt* gene was induced by less than 10 times of the control level. The molecular analysis revealed that the fraction of GC > TA transversions did not specifically increased. These results suggest that 8OHdG-mediated base substitutions do not mainly contribute to the mutagenic process involved in $KBrO_3$-induced carcinogenesis. Other genotoxic events must be involved in the carcinogenic process.

Our present studies strongly support this hypothesis. We demonstrated that $KBrO_3$ treatment clearly induced DNA damage in both the alkaline and neutral COM assay (Fig. 1). The alkaline COM assay is capable of detecting any DNA damages including DSB, single strand breaks (SSB), alkali-labile sites, DNA–DNA/DNA–protein cross-linking, and SSB associated with incomplete excision repair sites, while the neutral COM assay allows the detection of DSB, considered to be "biologically relevant" lesion of radiation damage [24]. $KBrO_3$ may have radio-mimic genotoxicity that yields oxidative DNA damage as well as DSB. $KBrO_3$ also induced MN formation and *TK* gene mutation significantly in TK6 cells. In the *TK* gene mutation assay, $KBrO_3$ predominantly produced SG mutants, but not NG mutants (Fig. 1c), implying that gross structural changes such as deletion and recombination are associated with the mutations. Molecular analysis of the *TK* mutants confirmed the assumption. Most of *TK* mutants showed LOH mutations, not non-LOH mutations, which are mainly point mutations. Harrington-Brock et al. also demonstrated that bromate compounds significantly induced *Tk* mutations in mouse lymphoma L5178Y cells, and almost all were LOH mutations [5]. LOH can be caused by deletions,