Available online at www.sciencedirect.com
ScienceDirect
Mutation Research 627 (2007) 31–35
Genetic Toxicology and Environmental Mutagenesis
www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

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

Brian Burlinson[a,*], Raymond R. Tice[b], Günter Speit[c], Eva Agurell[d], Susanne Y. Brendler-Schwaab[e], Andrew R. Collins[f], Patricia Escobar[g], Masamitsu Honma[h], Tirukalikundram S. Kumaravel[i], Madoka Nakajima[j], Yu F. Sasaki[k], Veronique Thybaud[l], Yoshifumi Uno[m], Marie Vasquez[n], Andreas Hartmann[o]

[a] *Huntingdon Life Sciences, Cellular & Molecular Toxicology, Woolley Road, Alconbury, Huntington, Cambs PE28 4HS, UK*
[b] *National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA*
[c] *Universitätsklinikum, Abteilung Humangenetik, Ulm, Germany*
[d] *Medical Products Agency, Uppsala, Sweden*
[e] *Federal Institute for Drugs and Medical Devices, Bonn, Germany*
[f] *Department of Nutrition, University of Oslo, Norway*
[g] *BioReliance, Invitrogen Bioservices, Rockville, MD, USA*
[h] *National Institute of Health Sciences, Tokyo, Japan*
[i] *Covance Laboratories Ltd., Harrogate, UK*
[j] *Bio Safety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan*
[k] *Hachinohe National College of Technology, Hachinohe, Japan*
[l] *Sanofi-Aventis, Vitry Sur Seine, France*
[m] *Mitsubishi Chemical Safety Institute Ltd., Shiba, Minato-ku, Tokyo, Japan*
[n] *Helix3 Inc., North Carolina, USA*
[o] *Safety Profiling & Assessment, Novartis Pharma AG, Basel, Switzerland*

Received 28 February 2006; received in revised form 31 July 2006; accepted 14 August 2006
Available online 22 November 2006

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

* Corresponding author. Tel.: +44 1480 893235.
E-mail address: burlinsb@ukorg.huntingdon.com (B. Burlinson).

1383-5718/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2006.08.011

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.
© 2006 Elsevier B.V. All rights reserved.

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.

References

[1] O. Ostling, K.J. Johanson, Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells, Biochem. Biophys. Res. Commun. 123 (1984) 291–298.

[2] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.

[3] R.R. Tice, P.W. Andrews, O. Hirai, N.P. Singh, The single cell gel (SCG) assay: an electrophoretic technique for the detection of DNA damage in individual cells, in: C.R. Witmer, R.R. Snyder, D.J. Jollow, G.F. Kalf, J.J. Kocsis, I.G. Sipes (Eds.), Biological Reactive Intermediates. IV. Molecular and Cellular Effects and Their Impact on Human Health, Plenum Press, New York, 1991, pp. 157–164.

[4] V.J. McKelvey-Martin, M.H. Green, P. Schmezer, B.L. Pool-Zobel, M.P. De Meo, A. Collins, The single cell gel electrophoresis assay (comet assay): a European review, Mutat. Res. 288 (1993) 47–63.

[5] D.W. Fairbairn, P.L. Olive, K.L. O’Neill, The Comet assay: a comprehensive review, Mutat. Res. 339 (1995) 37–59.

[6] R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.-C. Ryu, Y.F. Sasaki, The single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206–221.

[7] G. Speit, A. Hartmann, The comet assay—a sensitive genotoxicity test for the detection of DNA damage and repair, in: D.S.

Henderson (Ed.), Methods in Molecular Biology, vol. 113, DNA Repair Protocols: Eucaryotic Systems, 2nd ed., Humana Press Inc., Totowa, NJ, 2006, pp. 275–286.

[8] S.Y. Brendler-Schwaab, A. Hartmann, S. Pfuhler, G. Speit, The in vivo comet assay: use and status in genotoxicity testing, Mutagenesis 20 (2005) 245–254.

[9] Y.F. Sasaki, K. Sekihashi, F. Izumiyama, E. Nishidate, A. Saga, K. Ishida, S. Tsuda, The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and US NTP Carcinogenicity Database, Crit. Rev. Toxicol. 30 (2000) 629–799.

[10] A. Hartmann, E. Agurell, C. Beevers, S. Brendler-Schwaab, B. Burlinson, P. Clay, A.R. Collins, A. Smith, G. Speit, V. Thybaud, R.R. Tice, Recommendations for conducting the in vivo alkaline comet assay, Mutagenesis 18 (2003) 45–51.

[11] A. Hartmann, M. Schumacher, U. Plappert-Helbig, P. Lowe, W. Suter, L. Mueller, Use of the alkaline in vivo comet assay for mechanistic genotoxicity investigations, Mutagenesis 19 (2004) 51–59.

[12] COM (United Kingdom Committee on Mutagenicity of Chemicals in Food, Consumer Products, and the Environment), Guidance on a Strategy for Testing of Chemicals for Mutagenicity, COM, 2000.

[13] FDA, Guidance for Industry and Review Staff Recommended Approaches to Integration of Genetic Toxicology Study Results, FDA, 2006, http://www.fda.gov/cder/guidance/index.htm.

[14] OECD, Mammalian erythrocyte micronucleus test OECD Guidelines for the Testing of Chemicals, vol. 474, OECD, Paris, 1997 (test guideline).

[15] M. Hayashi, J.T. MacGregor, D.G. Gatehouse, D.H. Blakey, S.D. Dertinger, L. Abramsson-Zetterberg, G. Krishna, T. Morita, A. Russo, N. Asano, H. Suzuki, W. Ohyama, D. Gibson, In vivo erythrocyte micronucleus assay. III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test, Mutat. Res., this issue.

[16] Y.F. Sasaki, A. Saga, M. Akasaka, K. Yoshida, E. Nishidate, Y.Q. Su, N. Matsusaka, S. Tsuda, In vivo genotoxicity of orthophenylphenol, biphenyl, and thiabendazole detected in multiple mouse organs by the alkaline single cell gel electrophoresis assay, Mutat. Res. 395 (1997) 189–198.

[17] E.M. Bomhard, S.Y. Brendler-Schwaab, A. Freyberger, B.A. Herbold, K.H. Leser, M. Richter, *O*-Phenylphenol and its sodium and potassium salts: a toxicological assessment, Crit. Rev. Toxicol. 32 (2002) 551–626.

[18] P.L. Olive, J.P. Banath, Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent, Exp. Cell. Res. 221 (1995) 19–26.

[19] L. Henderson, A. Wolfreys, J. Fedyk, C. Bourner, S. Windebank, The ability of the comet assay to discriminate between gentoxins and cytotoxins, Mutagenesis 13 (1998) 89–94.

[20] A. Hartmann, E. Kiskinis, A. Fjaellman, W. Suter, Influence of cytotoxicity and compound precipitation on test results in the alkaline comet assay, Mutat. Res. 497 (2001) 199–212.

[21] M. Kiffe, P. Christen, P. Arni, Characterization of cytotoxic and genotoxic effects of different compounds in CHO K5 cells with the comet assay (single-cell gel electrophoresis assay), Mutat. Res. 537 (2003) 151–168.

[22] M.S. Rundell, E.D. Wagner, M.J. Plewa, The comet assay: genotoxic damage or nuclear fragmentation? Environ. Molec. Mutagen. 42 (2003) 61–67.

[23] T. Mensing, P. Welge, B. Voss, L.M. Fels, H.H. Fricke, T. Bruning, M. Wilhelm, Renal toxicity after chronic inhalation exposure of rats to trichloroethylene, Toxicol. Lett. 128 (2002) 243–247.

[24] G. Scassellati-Sforzolini, R. Pasquini, M. Moretti, M. Villarini, C. Fatigoni, P. Dolara, S. Monarca, G. Caderni, F. Kuchenmeister, P. Schmezer, B.L. Pool-Zobel, In vivo studies on genotoxicity of pure and commercial linuron, Mutat. Res. 390 (1997) 207–221.

[25] Y.F. Sasaki, A. Saga, M. Akasaka, S. Ishibashi, K. Yoshida, Y.Q. Su, N. Matsusaka, S. Tsuda, Detection of in vivo genotoxicity of haloalkanes and haloalkenes carcinogenic to rodents by the alkaline single cell gel electrophoresis (Comet) assay in multiple mouse organs, Mutat. Res. 419 (1998) 13–20.

[26] G.H.S. Strauss, Non-random cell killing in cryopreservation: implications for performance of the battery of leukocyte tests (BLT). I. Toxic and immunotoxic effects, Mutat. Res. 252 (1991) 1–15.

[27] M. Vasquez, R.R. Tice, Detecting genotoxic activity against high molecular weight DNA using the alkaline single cell gel (SCG) assay, Environ. Molec. Mutagen. 29 (1997) 53.

[28] A.R. Collins, A. Ai-guo, S.J. Duthie, The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells, Mutat. Res. 336 (1995) 69–77.

[29] P.L. Olive, J.P. Banath, R.E. Durand, Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the “Comet” assay, Radiat. Res. 122 (1990) 86–94.

[30] T.S. Kumaravel, A.N. Jha, Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals, Mutat. Res. 605 (2006) 7–16.

[31] S.J. Wiklund, E. Agurell, Aspects of design and statistical analysis in the comet assay, Mutagenesis 18 (2003) 167–175.