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Automated analysis of DNA damage in the high-throughput version of the comet assay

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ABSTRACT

Recently a high-throughput version of the comet assay was developed using a special 96-well multi-chamber plate (MCP) [1]. In this version, the electrophoresis is performed directly on the MCP, which makes transferring of cells to microscope slides unnecessary.

In order to facilitate the scoring procedure we adapted an automated slide-scanning system (Metafer MetaCyte with CometScan) to enable unattended analysis of comets on the MCP. The results of the system were compared with the data obtained with two interactive comet-assay analysis systems. For induction of DNA damage in human fibroblasts methylmethane sulfonate (MMS) or H_2O_2 was used. The three systems revealed similar, concentration-dependent results for all parameters tested: tail moment (tm), % DNA-in-tail and olive tail moment. Near the detection limit of 5–6% DNA-in-tail a significant difference with the untreated control was obtained by use of four parallel samples ($p = 0.01$). With the newly developed automated analysis system, the evaluation of either 50 or 100 comets yielded similar standard errors for either treatment with MMS or H_2O_2, thus showing that the method is suitable to reveal the crucial low-dose effects with high precision. The results also show that the time needed for automated evaluation of comets on the MCP was reduced by a factor of 10 when compared with the time required for interactive evaluation. In summary, the high-throughput version of the comet assay combined with the automated evaluating system increased the output by a factor up to 180 compared with the standard method.

1. Introduction

Bio-monitoring of environmental probes, or examining the genotoxic potential of chemicals according to REACH (registration, evaluation and authorisation of chemicals), or pre-screening of pharmaceutical candidate compounds requires the measurement of large numbers of samples. Therefore, a high-throughput method for mammalian genotoxicity testing is desirable.

For determining DNA damage, the comet assay is a well-established genotoxicity test that offers the possibility of measuring in a high-throughput mode. The comet assay allows testing of a broad spectrum of DNA damage with high sensitivity, *in vitro* as well as *in vivo* [3,4]. The comet assay was first introduced by Östling and Johanson [5] and was further refined by a number of laboratories. Singh et al. [6] developed the more versatile alkaline method of the comet assay. Based on this assay and the guidelines of Tice et al. [7] a high-throughput version of the conventional comet assay was recently developed [1,2]. This method allows testing of 96 samples at one time by using a modified 96-well plate (multichamber plate, MCP). The innovation of the MCP makes it possible to perform the electrophoresis directly on the plate, without transferring the cells to slides [1,2].

Comparison of the conventional comet assay with the high-throughput version revealed similar results [1]. Directly as well as indirectly acting mutagens could be measured with high sensitivity. After metabolic activation with rat-liver S9 fraction cyclophosphamide, benzo(a)pyrene [11], and pentachlorophenol [12] induced comet formation in a concentration-dependent way.

So far, the evaluation of DNA damage in the comet assay is a very time-consuming step, which is done by microscopic fluorescence-analysis of individual comets, thus taking several hours for each single experiment. In the past, some automated analyzing systems were developed for the conventional assay [8–10], which reduced the comet scoring time by approximately 50% compared

Abbreviations: D-MEM, Dulbecco's modified eagle medium; D-PBS-buffer, Dulbecco's phosphate-buffered saline buffer; FDA, fluorescein diacetate; MCP, multichamber plate; MMS, methylmethane sulfonate; REACH, registration, evaluation and authorisation of chemicals; SD, standard deviation; SE, standard error; tm, tail moment.

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with manual evaluation, and made unattended overnight evaluation possible.

To analyze the comets on the MCP we developed a method to score comet-assay samples using the fully automated slide-scanning platform Metafer and the MetaCyte CometScan software. In this publication we present data from the comparison of scan results obtained by automated analysis with the results obtained with two interactive comet-assay analysis systems. We measured the genotoxic effects of two DNA-damaging chemicals, methylmethane sulfonate (MMS) and hydrogen peroxide (H_2O_2) in human fibroblasts.

2. Materials and methods

2.1. Cell cultures

Human fibroblasts from the cell line NHDF-p were purchased from Promochem (Heidelberg, FRG). The cells were grown in D-MEM, supplemented with 12% fetal calf serum, vitamins, non-essential amino acids, 100 U/ml of penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2 and 95% air with more than 95% humidity. The human fibroblasts were used in passage 8–15.

2.2. Multichamber plate (MCP)

The MCP is a specially coated 96-well plate purchased from Intox, Oldenburg, FRG. This coating and its specific design make it suitable for the electrophoresis of cellular DNA. The surrounding walls of the wells can be separated from the flat base plate of the MCP. Cultured cells adhere to the coated layer and are maintained on the MCP throughout the whole comet-assay procedure. All steps can be performed together for 96 samples, inclusive electrophoresis after detaching the walls from the MCP plate [2].

2.3. Chemical treatment of the cells

Cells were treated with methylmethane sulfonate (MMS, >99%; Sigma, Deisenhofen, FRG), or H_2O_2 (37% aqueous solution; Acros Organics, NJ). MMS was freshly dissolved in serum-free medium at pH 7.2 and 37 °C directly before cell treatment. H_2O_2 was also diluted in serum-free medium immediately before cell treatment. The cells were exposed to MMS for 1 h; the treatment time was reduced to 15 min for H_2O_2 because of its short half-life. The test concentrations that we used resulted in DNA damage from 0–90% for single comets within the sample. The highest concentrations were non-toxic, as measured by the fluorescein diacetate assay (data not shown).

2.4. Comet assay in the high-throughput version (MCP)

The comet assay in the high-throughput version was performed on the basis of the guidelines of Tice et al. [7], described by Stang and Witte [1] in detail. Before seeding the cells, the base plate of the MCP was covered with poly-L-lysine for 30 min and washed three times with D-PBS-buffer. A total of 3000–5000 cells were seeded into each well of the MCP. The adherent cells were allowed to attach to the bottom of the MCP for 4 h. Afterwards the MCP was centrifuged (Labofuge 400; rotor: 8177; Heraeus®, FRG) for 2 min at 120 × *g*. The cells were washed with serum-free medium, treated with the genotoxic chemicals as described above, and centrifuged again. The genotoxic chemicals were removed, and the walls surrounding the MCP were separated from the plate. The plate with the cells was covered with a solution of 0.5% low-melting agarose (type Sea Plaque agarose from Biozym Diagnostik, Hessisch Oldendorf, FRG) preheated to 37 °C. The plate was kept in the refrigerator for 5 min to solidify the low-melting agarose. Subsequent steps were performed according the procedure recommended by Tice et al. [7] and described in Stang and Witte [1].

Briefly, the plate was covered with refrigerated lysis solution, pH 10.0, and kept at 4 °C for 1 h. After lysis the plate was placed on a horizontal electrophoresis box. The box was filled with freshly prepared alkaline buffer whereby the plate was completely covered with the buffer. During 40 min at 4 °C the alkaline treatment allowed alkaline unwinding of the DNA and DNA breakage at alkali-labile sites. After electrophoresis (300 mA, 25 V, 20 min) the plate was covered three times with neutralization buffer, washed with aqua bidest and stained with SYBR Green (Sigma, Deisenhofen, FRG). Fluorescence microscopy was used to determine the median DNA migration in each of up to 12 parallel samples per concentration (100 comets/well). The mean, the standard deviation (SD), and the standard error (SE) of the medians of the parallel samples were calculated. Statistical significance of the differences between the DNA migration induced by low concentrations of the mutagens and the untreated controls was determined by the non-parametric Mann-Whitney test and the parametric *t*-test if the basic assumptions for parametric tests were met.

2.5. Automated comet-assay analysis

Automated analysis of the comet assay was performed by use of a MetaCyte CometScan system based on the slide-scanning platform Metafer (MetaSystems, Altlussheim, FRG). This commercially available system consists of a motorized microscope (AxioImager Z1, Carl Zeiss, Jena, Germany) with fluorescence illumination, a motorized X/Y scanning stage (Maerzhaeuser, Wetzlar, Germany) with a range of 225 × 76 mm, a high-resolution monochrome megapixel charge-coupled device (CCD) camera (M4+; JAI AS, Glostrup/Copenhagen, Denmark), and a Windows™ compatible PC (DELL, Langen, Germany) running the Metafer software. Hardware components (e.g. the microscope-focusing motor, the fluorescence filter-turret and the motorized stage) are directly driven by the software. The Maerzhaeuser stage has been modified to adapt it to the 96-well plates used in this study. Parameters for slide-scanning and automated comet-assay analysis were set according to the experiment's requirements by selecting the matching settings file (classifiers) in the Metafer software. Classifiers define details on image acquisition, number of captured fields at each well position, cell selection, image-analysis procedures, and more. In addition, layout-setting files defining the number and positions of wells to scan on the plate were created.

To start the analysis, the classifier and layout files were selected in the set-up dialogue of the Metafer software, and a file name for the results file was entered. Unattended MCP scanning was started subsequently using a final magnification of 10×. The plane of best focus was determined automatically at each captured field inside the wells. This is done by automatically moving the stage in the *z*-direction, capturing images in different focal planes, and analyzing the focus quality based on a local contrast criterion. Subsequently the exposure time for the final image was automatically adjusted, avoiding saturated pixels. Each image was then analyzed for the presence of target comet cells. Target cells are defined in the classifier by morphology criteria such as size, aspect ratio, concavities, and other parameters.

Cells being initially detected by the system were rejected if they met the following conditions: (a) another object was present in the close neighborhood, that might interfere with the measurements, (b) the background around the candidate comet showed significant inhomogeneity, and (c) the tail intensity of the comet did not decrease to the background level within the measurement rectangle, indicating that the comet is larger than the region of interest. Heavily damaged cells with no clearly detectable heads are automatically excluded from analysis, too. The software either correctly detects a missing head, or erroneously identifies the tail region as a large head. Both situations can be recognized and used either for rejection of the cell, or for classifying the cells as objects for further inspection.

Once a comet was finally accepted by the system, its intensity profile was automatically analyzed within a measurement rectangle defined by the software. Head and tail of the comet were determined based on the intensity levels. The background levels were subtracted from the intensity values obtained. Different comet features (e.g. intensity of head and tail, comet shape, tail moment) were measured, and an image of each cell was stored in a gallery. Overlays within these cell images show borderlines between head and tail, and the head and tail regions, as they were defined by the analysis algorithms. Depending on the classifier set-up, selected cell features (e.g. tail moment and percentage of DNA in the tail) were displayed in the gallery image.

2.6. Manual comet-assay analysis

Samples analyzed with the MetaCyte CometScan Software were subsequently analyzed with conventional evaluation systems. Each sample on the MCP was interactively evaluated using the Lucia Comet-Assay Single-Stain software (Laboratory Imaging s.r.o, Czech Republic), which is a separate stand-alone imaging system, and the MetaSystems CometImager, which represents the interactive evaluation system of MetaCyte CometScan. The MetaSystems CometImager uses the same imaging hardware and scoring algorithms as Metafer. For the interactive evaluation 100 comets/well were randomly selected and measured.

3. Results

To compare the newly developed automated comet analysis system with two manual (interactive) analysis systems we evaluated the DNA damage of identical samples. Human fibroblasts were treated with MMS for 1 h or with H_2O_2 for 15 min on the MCP. In the untreated control samples no or only a very low DNA migration was observed. The results of the commonly used parameters tail moment (tm) and % DNA-in-tail are shown in Fig. 1 for MMS and in Fig. 2 for H_2O_2.

The comet formation for both chemicals showed concentration-dependent induction of DNA damage. The quantitative results were similar for automatic and interactive measurements both for MMS (Fig. 1) and for H_2O_2 treatment (Fig. 2). They were also similar regarding the parameter tm or % DNA-in-tail. The Olive tail