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A NOVEL PARAMETER IN COMET ASSAY MEASUREMENTS

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Single Cell Gel Electrophoresis (SCGE) or Comet assay is a very sensitive method for assessing damages in DNA on a single cell level. It has found many applications in fields where genotoxic activity could be an issue. In environmental monitoring, health care, food industry Comet assay is used with increasing popularity. For verifying the results obtained by this method many parameters could be monitored.To that end several software packages exist. In the conditions that we are suggesting one more parameter could be measured – comet shape. We argue that this parameter could be an advantage of the Comet Assay when the way of DNA damaging needs to be predicted.

Key words: Comet Assay, apoptosis, micrococcal nuclease

INTRODUCTION

Single – Cell Gel Electrophoresis (SCGE or Comet Assay) was first described by ÖSTLING and JOHANSON (1984) under neutral conditions and by SINGH

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et al. (1988) and OLIVE (1990) under alkaline conditions, as a simple protocol for determining genotoxic events in single cells. With its simplicity, sensitivity, speed and economy the assay has become one of the standart methods for assessing DNA damage with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, as well as fundamental research in DNA damage and repair (COLLINS, 2004; MCKELVEY-MARTIN *et al.,* 1993; FAIRBAIRN *et al.,* 1995; ROJAS *et al*., 1999). Briefly, Comet Assay includes the following steps: embedding of cells in agarose, lysis, electrophoresis, staining with a fluorescent DNA dye and visualization under fluorescent microscope. The electric field during the electrophoretic stage forces the damaged DNA out of the cell nucleus which leads to a characteristic comet-like appearance. Usually a comet consists of a head, this is the residual nucleus, representing the non-damaged DNA, a halo and a tail, formed by the damaged DNA. Changing pH conditions during electrophoresis helps to discriminate between different DNA damages: the neutral assay identifies double-strand breaks (ÖSTLING and JOHANSON 1984), while the alkaline variant detects single-strand, double-strand DNA breaks, alkali-labile sites (ALS) and DNA-DNA / DNA-protein cross-linking (SINGH *et al*., 1988; OLIVE *et al*.,1990). Having in mind that almost all genotoxic agents induce predominantly single-strand DNA breaks and/or ALS, the alkaline version of the assay is more sensitive for identifying genotoxic agents (TICE *et al*., 2000). An expert panel has designed recommendations for performing the Comet assay as an effort to standartize it. However, the same panel stated that other conditions should be applied when Comet Assay is aimed to fulfill specific requirements (TICE *et al*., 2000; HARTMANN *et al*., 2003).

There are several methods for quantifying the damaging potential of a given chemical agent by the method Comet Assay. The simplest one determines the proportion of cells with tails; another one classifies the comets into several categories, where DNA migration could be calculated by assigning a numerical value to each migration class (GEDIK *et al*., 1992; KOBAYASHI *et al*., 1995). Tail moment TM is a more elaborate measurement which includes the length of the comet tails and an intensity parameter (OLIVE *et al*., 1990). There are many specialized software packages that greatly facilitate comet calculations. The most commonly used parameters are tail length, relative fluorescence intensity of head and tail and tail moment. At least more than 40 different parameters can be performed.

Micrococcal nuclease (MNase) was first described by CUNNINGHAM *et al*., who in 1956 recovered a deoxyribonuclease from culture supernatant of the bacterium *Staphylococcus aureus*. This extracellular nuclease has a specificity for DNA cleavage in the linker region between nucleosomes, which has led to its extensive use in chromatin studies (NOLL, 1974). Limited micrococcal nuclease digestion of chromatin results in the well-known nucleosome ladder (TELFORD and STEWART, 1989). On the contrary, hydrogen peroxide, which causes hydroxyl radical-induced DNA cleavage, is not restrained by cellular structure and is quite independent of the packaging of DNA into chromatin. It is often used as a reporter in

Comet Assay applications (MCCARTHY *et al.*, 1997). Both H_2O_2 and MNase hydrolyse DNA in an extensively different manner.

The programmed cell death, called apoptosis is controlled by a genetic mechanism (FAIRBAIRN *et al.*, 1996). One of the most characteristic biochemical phenomena of apoptosis induced cells is DNA fragmentation to 180-200 bp by endonucleases in the linker region between nucleosomes (OLIVE *et al.*, 1995), which produces micrococcal nuclease-like DNA ladder pattern. It is considered that Comet Assay could be used for detecting DNA fragmentation in apoptotic cells and it is acknowledged that apoptotic cells exhibit specific comet appearance (OLIVE and BANATH, 1995; GODARD *et al.*, 1999a; FLORENT *et al.*, 1999; CHOUCROUN *et al.*, 2001). However, comet characteristic such as the general comet appearance and shape have received little or no attention. We consider that comet shape is another one parameter which can be additionally used in Comet Assay calculations. We called it S_c which means shape of a comet. Furthermore, we think that the shape of a comet gives us useful information about how different chemical agents act on the molecule of DNA. In this study we compared various comet shapes, obtained by different DNA cutting agents. Here, we argue that by otherwise uniform Comet Assay conditions the mode of action of the agent affects the comet appearance in a unique way, suggesting comet shape application in Comet Assay measurements.

MATERIALS AND METHODS

Cell culture - K 562 (ATCC) cells were derived from human erythroleukemia and were grown in RPMI 1640 medium (Sigma), supplemented with 10 % bovine serum to a concentration of approximately 10^6 cells/ml at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For each experiment cell viability was tested using cell viability trypan blue test.

TREATMENT WITH DNA CUTTING AGENTS

H_2O_2 treatment - ells were suspended in a phosphate-buffered saline 1 x PBS (2.68mM KCl, 1.47mM KH_2PO_4, 1.37mM NaCL, 8mM Na_2HPO_4; pH 7) to a final density of 1.6x 10^6 cells/ml and were treated with increasing concentrations of H_2O_2 for 10 minutes on ice.

Micrococcal nuclease treatment - Cells were resuspended in 1 x PBS, pH 7. Aliquotes of 50 µl cells were mixed with 50 µl 1.4% low-gelling agarose type VII (Sigma). The agarose-cell suspension was quickly layered on a precoated with normal agarose type II-A (Sigma) glass slide and covered with a coverslip to allow the gel to spread. Incubation on ice for 5 min was performed. After that coverslips were removed. 100 µl of micrococcal dilutions (MBI Fermentas) in MNase buffer (M Sorbitol, 5mM $CaCl_2$, 10 mM Tris-HCl, pH 8, 5 mM NaCl) were applied on the surface of the gels, covered with coverslips and incubated at 37°C for 1 min. The enzyme reaction was stopped by immersing the slides into lysis solution.

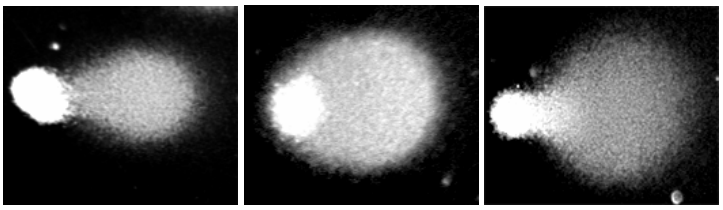
Apoptosis induction - Exponential cultures (4 x 10^5 cells/ml) were treated with 200 ng/ml Actinomycin D (Sigma) for 2 hours at 37°C (KRESSEL and GROSCURTH, 1994; ZHANG *et al*., 2000). Cells were washed with 1 x PBS, resuspended in fresh medium and incubation continued. Aliquotes were taken at 2, 4, 6, 8 and 10 hours after Actinomycin D addition. Apoptosis was visually monitored by counting the cells with blebbing membrane (CHOUCROUN *et al*., 2001) and by FACS analysis (GODARD *et al*., 1999b). The percentage of cells directed to apoptosis increased gradually from 20% at 2 hours after induction and reached 95-100% at 10 hours post induction.

Comet Assay - We performed the alkaline variant of Comet Assay, modified by OLIVE *et al*., 1989. After preparing the gels, spread on the glass slides, the latter were immediately placed in a cold lysis solution (1M NaCl, 50 mM EDTA, pH 8, 30 mM NaOH, 0.1 % N-lauroylsarcozine; pH 10) for 1 hour. DNA was denatured by incubating the slides 3 x 20 minutes in a denaturing solution (30 mM NaOH, 10 mM EDTA, pH 8; pH 12.6). Electrophoresis was conducted at 10°C for 20 minutes, 0.45 V/cm in an electrophoresis buffer (30mM NaOH, 10 mM EDTA, pH 8; pH 12.6). The glass slides were then washed with 0.5 M Tris-HCl, pH 7.5 to neutralize the alkali and were dehydrated by sequential washes in 75 and 95% ethanol for 5 min each.

The comets in the gel were stained with 0.5 μg/ml ethidium bromide and visualized by a fluorescent microscope. About 100 comets were scored and experiments with every DNA cutting agent were repeated at least three times.

RESULTS AND DISCUSSION

Comet Assay is a technique for detecting fragmented DNA at the level of a single cell. Different DNA cutting agents have diverse mode of action. Therefore, we decided to test whether the action of MNase and H_2O_2 on K 562 cells will show differences using Comet Assay. Both agents cleave DNA in a specific mannner: micrococcal nuclease cut DNA preferentially in the linker region between two nucleosomes, while H_2O_2 causes hydroxyl radical-induced DNA cleavage resulting in random infliction of DNA breaks.



MNase H_2O_2 Apoptosis

Fig. 1. Comets obtained by Comet Assay using different substances

In our experiments we used the alkaline variant of the method in agreement with its broadly accepted increased sensitivity (TICE *et al*., 2000) for identi-

fying agents with genotoxic activity. K 562 cells were treated with increasing concentrations of MNase or H_2O_2 and then subjected to Comet Assay. (Fig. 1**,** MNase, H_2O_2) shows typical comet images of DNA migrated toward the anode during the electrophoretic stage of the assay. The difference between comets, produced by MNase and H_2O_2 is clearly visible. The comets, obtained by MNase, display a typical "bat-like" shape, while H_2O_2 –obtained ones resemble balloons. As digestion proceeds these features are maintained and magnified. We have taken extra precausions to optimize the Comet Assay in order to consistently obtain slides with uniform comet populations. More than 90% of the comets were similar in size and shape. The differences of the comet shape, obtained with the two agents were reproducible in all our experiments.

In order to prove our statement that comet shape is different for different DNA cutting agents, we performed Comet Assay on cells, induced to apoptosis. It is considered that Comet Assay is a method that could be used for detection of DNA fragmentation in an apoptotic cell (OHYAMA *et al.*, 1998). A well-known feature of the programmed cell death is the so-called "laddering" of genomic DNA (STEWART, 1994). When analyzed on agarose gels this digestion pattern produces a picture identical to chromatin digested with MNase (ALLEN *et al.*, 1997). In our experiments K 562 cells were treated with apoptosis-inducible chemical Actynomycin D (KRESSEL and GROSCURTH, 1994; ZHANG *et al.*, 2000) and then subjected to Comet assay. (Fig. 1**,** Apoptosis) shows comets, obtained from the apoptotic cells. It is easily seen that apoptotic comets resemble those of MNase and are quite different in comparison with H_2O_2 –obtained ones. They have a characteristic image, which is called by others a pear-shaped, a teardrop, or large-fan like tail (BOCK *et al.*, 1999; KIZILIAN *et al.*, 1999; OLIVE *et al.*, 1995; WILKINS *et al.*, 2002). It is considered that this spread migrated DNA is indicative of apoptotic cells.

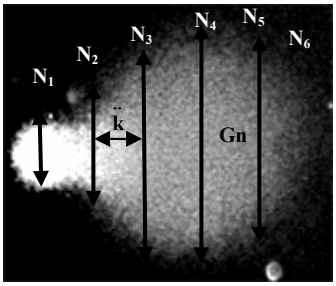


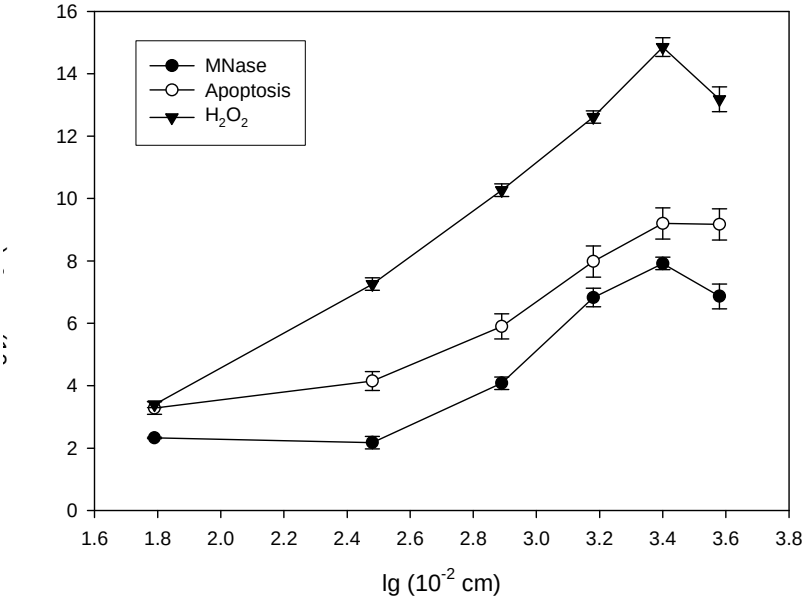
Fig. 2. Comet shape could be mathematically calculated. The image of a comet was separated into equally distributed grids. The longitude of each grid was measured and the shape was calculated by the equation *Sc= Gn.lgN.k* (for details see text)

For mathematically verifying the statement that comet shape is a novel, additional and informative parameter in Comet Assay measurements we first divided the comets, obtained with MNase and H_2O_2, into several classes regarding

their lengths (Fig. 2) . It is considered that this is a standart method for determining DNA damaging potential of a tested substance (GEDIK *et al*., 1992; KOBAYASHI *et al*., 1995). By using photoshop 6.0 software programme we divided the comets of the same class into several equally (*k*) separated perpendicular grids (*N*). After that the length of grids (*Gn)* was measured by the means of the programme. According to us, by calculating the so-called S_C (shape of a comet) which represents the length of the separate grids in the confine of a comet, we could easily differentiate between different shapes of comets, which could be expressed by the equation:

$$S_C = Gn.lgN.k,$$

where *Gn* means length of grid n;
N – number of a grid;
k – distance between different grids, which is a constant;



Graphic 1. Comet shape represented by the equation: *Sc=Gn.lgN.k*

Graphic 1 is built by measuring the comet shapes (*Sc'*s), obtained by H_2O_2, MNase and apoptosis. It definitely shows the differences between comet shapes. Each curve on the chart connects 7 separate points, each representing the parameter *Sc* for a separate grid, encompassing the boundary of the observed comet. H_2O_2 and MNase curves present a well seen difference, which can be explained by the different mode of action of the two agents.

Our results show that comet shapes of apoptotic cells resemble those of MNase ones (Fig. 1, compare MNase with Apoptosis). Graphic 1 unambiguously

proves this statement. The curve of apoptotic comet shapes resembles the one with MNase-obtained ones. This result could be explained by the action of several MNase-like nucleases responsible for DNA internucleosomal cleavage in the process of apoptosis.

In this paper we strongly argue that the Comet shape (*Sc*) is an informative parameter for the mode of action of the tested substances. Comet Assay is a relatively new method for assessing DNA damage at the level of a single cell. Comet scoring and data maintaining are still being discussed. A lot of different parameters can be measured for determining of genotoxic chemicals. In this paper we state that a novel parameter called comet shape (*Sc*) could be used for differentiating between diverse mode of action of DNA cutting agents. Comet shape could bring an additional, useful information about DNA damage specificity.

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NOVI PARAMETRI MERENJA U EKSPERIMENTU PRIMENE METODA ELEKTROFOREZE POJEDINAČNIH ĆELIJA (SCGE)

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I z v o d

Elektroforeza pojedinačnih ćelija (SCGE) ili Comet eksperiment je veoma osetljiva metoda za ispitivanje oštećenja DNK na nivou pojedinačnih ćelija. Ova metoda je našla primenu u oblasti genotoksikoloških ispitivanja. U ispitivanju uslova spoljne sredine, zdravlja, industrije hrane ovaj metod postaje sve popularniji. Za verifikaciju rezultata ispitivanja korišćenjem ove metode mogu se utvrditi i koristiti mnogi parametri. Do sada je razvijeno nekoliko paketa kompjuterskih programa. U uslovima koje predlažemo dodatni parametar može da se koristi – elektroforegrami pojedinačnih ćelija. Smatramo da ovaj parametar ima prednosti u eksperimentima u kojima je potrebno predvideti način oštećenja DNK.

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