Use of Single Cell Gel Electrophoresis (Comet Assay) Modifications for Analysis of DNA Damage

E. HORVÁTHOVÁ, D. SLAMEŇOVÁ AND A. GÁBELOVÁ

Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia

Abstract. Single cell gel electrophoresis (SCGE) or comet assay is a rapid and sensitive fluorescent microscopic method which allows measurement of DNA strand breaks in individual cells. Modifications of SCGE conditions permitted to detect different types of DNA damage. In order to characterize DNA damage induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and hydrogen peroxide (H_2O_2) in Chinese hamster V79 cells, two approaches were used: **(1)** two pH values of unwinding and electrophoresis solutions (pH $\geq$ 13.0 and pH = 12.1) to specify the type of DNA lesions {the alkali-labile sites and true DNA single-strand breaks (ssb)} and **(2)** DNA glycosylases {endonuclease III (EndoIII) and formamidopyrimidine-DNA glycosylase (FaPy)} or DNA inhibitors {hydroxyurea (HU) + 1-(β-D-arabinofuranosyl)cytosine (AraC)} to characterize the types of DNA damage. Our results showed that the lesions induced by H_2O_2 represented mainly the true DNA ssb, while MNNG formed predominantly alkali-labile sites, which were converted to DNA ssb under strong alkaline conditions (pH $\geq$ 13.0). The effects of DNA repair enzymes and DNA inhibitors were more significant under lower pH (pH = 12.1) of unwinding and electrophoresis solution. Both, DNA glycosylases and DNA inhibitors increased the level of DNA ssb.

Key words: Hamster cells V79 — Comet assay — Repair enzymes — Alkylating agent MNNG — Hydrogen peroxide H_2O_2

Introduction

Single cell gel electrophoresis (SCGE) or comet assay is a rapid and sensitive fluorescent microscopic method which allows measurement of single-strand (ss) DNA breaks at low levels of damage in individual cells (Östling and Johanson 1984; Singh et al. 1988; Collins et al. 1995). Ss DNA breaks result from a number of different types of reactions in cells (Eastman and Barry 1992). However, standard methods for measuring ss breaks, including the comet assay, do not indicate the

Correspondence to: Eva Horváthová, Cancer Research Institute, Slovak Academy of Sciences, Department of Mutagenesis and Carcinogenesis, Vlárska 7, 833 91 Bratislava, Slovakia. E-mail: exonhore@savba.sk

origin of these breaks. Generally, the monofunctional alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) causes instability of N-glycosyl bond of DNA, resulting in the appearance of alkali-labile sites and apurinic/apyrimidinic (AP) sites in DNA (Singer 1986) In contrast to MNNG, hydrogen peroxide (H_2O_2) induces formation of highly reactive hydroxyl radicals $^{\bullet}OH$, which attack DNA and create strand breaks, AP sites and base modifications in DNA (Halliwell and Aruoma 1991) We attempted to increase the predictive value of the comet assay for characterisation of the nature and origin of ss DNA breaks induced by MNNG and H_2O_2 in Chinese hamster V79 cells by two steps. (1) parallel use of two pH values for unwinding and electrophoresis, namely pH = 12.1 and pH $\geq$ 13.0, as generally alkali-labile sites are stable until pH is raised to pH = 12 5 (Fortini et al 1996), (2) use of DNA glycosylases {endonuclease III (EndoIII), specific for oxidised pyrimidines and formamidopyrimidine-DNA glycosylase (FaPy), specific for damaged purines (Epe et al. 1993)} or DNA inhibitors {hydroxyurea (HU) + 1-(β-D-arabinofuranosyl)cytosine (AraC)}

Materials and Methods

Cell culture Quasidiploid Chinese hamster lung fibroblasts V79 were obtained from Prof A Abbondandolo, Laboratory of Mutagenesis, National Institute for Cancer Research, Genova, Italy Cells were grown at 37 °C in humidified atmosphere of 5% CO_2 in Eagle's MEM supplemented with 6% foetal calf serum and antibiotics (penicillin 100 U/ml, streptomycin and kanamycin 100 μg/ml)

Chemicals and treatment of cells N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich, Germany) stock solution in DMSO (60 mmol/l) was kept at -20 °C and diluted immediately before use Hydrogen peroxide (Lachema, Brno, Czech Republic) stock solution (10 mol/l) was kept at 4 °C and diluted immediately before use in PBS buffer (Ca^{2+}- and Mg^{2+}-free) at 4 °C

Exponentially growing cells were treated either with MNNG (0 03, 0 06, 0 12 mmol/l – 120 min in coplete medium) or with H_2O_2 (25, 50, 100 μmol/l – 5 min at 4 °C in Ca^{2+}- and Mg^{2+}-free PBS buffer) Inhibitors, hydroxyurea (HU) and 1-(β-D-arabinofuranosyl)cytosine (AraC) (Sigma, St Louis, USA), were applied during 120 min treatment of cells with MNNG at final concentrations of 2×10^{-3} mol/l and 2×10^{-5} mol/l, respectively

Single cell gel electrophoresis (comet assay) The procedure of Singh et al (1988) was used with minor modifications of Slameňová et al (1997) and Gábelová et al (1997) Control or treated V79 cells embedded in agarose were immersed in ice-cold lysis solution (2 5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, pH 10 0, 1% Triton X-100) for 60 min In experiments investigating the nature of induced damage, slides were after lysis washed 3 times for 5 min in endonuclease buffer (40 mmol/l HEPES-KOH, 0 1 mol/l KCl, 0 5 mmol/l EDTA, 0 2 mg/ml BSA, pH 8 0) and incubated at 37 °C for 45 min with endonuclease III (EndoIII), for 30 min with formamidopyrimidine-DNA glycosylase (FaPy) or with buffer alone EndoIII and FaPy were obtained as crude extracts from Dr A R Collins (Aberdeen) Then the slides were placed in electrophoresis boxes containing alkaline solutions pH $\geq$ 13 0 (0 3 mol/l NaOH, 1 mmol/l Na_2EDTA) or pH = 12 1 (0 03 mol/l NaOH, 1 mmol/l Na_2EDTA) for 40 min at 4 °C Electrophoresis was carried out for 30 min at 4 °C The slides were then neutralised with 0 4 mol/l Tris-HCl, pH 7 5, for three 5-min washing steps and stained with 20 μl ethidium bromide (10 μg/ml) "Comets" were examined using a Zeiss epifluorescence microscope attached to an intensifying solid state

CCD camera and image analysis system (Komet 3 0, from Kinetic Imaging Ltd) The "% of tail DNA" was used as the measure of DNA damage In the experiment 200 comets were scored per each sample The significance of differences between samples was assessed by Student's *t*-test

Results

Discrimination between alkali-labile sites (pH ≥ 13.0) and ss DNA breaks (pH = 12.1). The levels of induced ss DNA breaks in MNNG- and H_2O_2-treated V79 cells are shown in Fig. 1. The levels of damage were measured immediately after treatment of cells with MNNG and H_2O_2 to minimize removal of DNA lesions by cellular repair.

Characterisation of MNNG-induced damage by repair enzymes (EndoIII, FaPy) and inhibitors (HU + AraC) using two parallel pH values, pH ≥ 13.0 and pH = 12.1, for unwinding and electrophoresis. The influence of DNA repair enzymes and DNA inhibitors on MNNG-treated V79 cells under standard SCGE alkaline conditions (pH ≥ 13.0) is shown in the left part of Fig. 2. The effects of DNA repair enzymes and DNA inhibitors become evident under the decreased pH = 12.1 of unwinding and electrophoresis solution, which does not enable the detection of alkali-labile lesions in DNA (right part of Fig. 2).

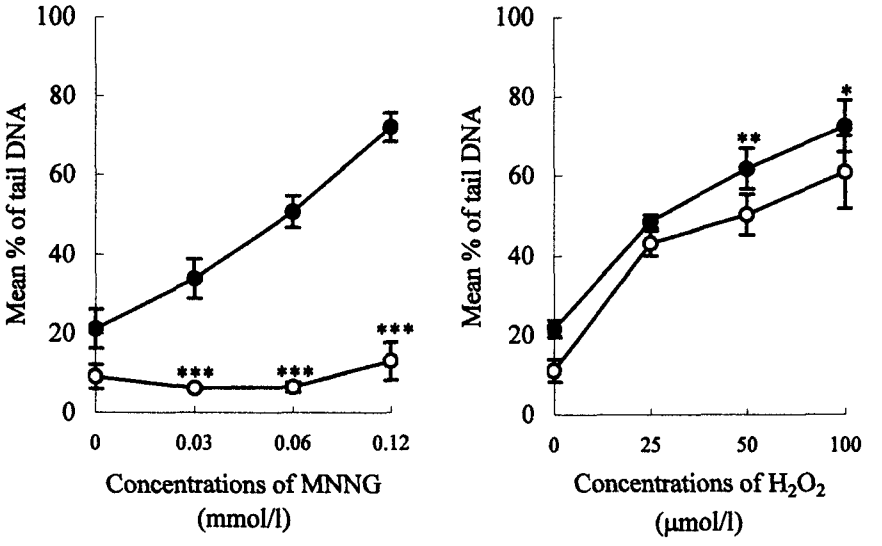

Figure 1. Levels of induced ss DNA breaks in MNNG- (left) and in H_2O_2-treated V79 cells (right) under standard SCGE alkaline conditions (• pH ≥ 13 0) and under decreased o pH = 12 1 of unwinding and electrophoresis solution The results represent the mean of two independent experiments ± SD value Statistically significant differences $*p \leq 0\ 05$, $**p \leq 0.01$, $***p \leq 0\ 001$ represent the difference between values measured at pH ≥ 13 0 and 12.1

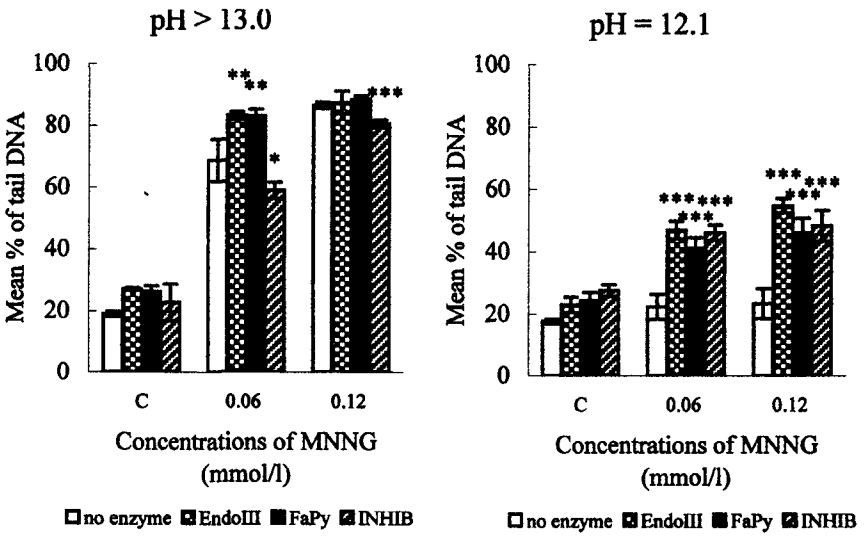

Figure 2. Influence of DNA repair enzymes {EndoIII and FaPy} and inhibitors {HU (2×10^{-3} mol/l) + AraC (2×10^{-5} mol/l)} on MNNG-treated V79 cells under standard SCGE alkaline conditions (pH $\geq$ 13.0) (left) and under decreased pH = 12.1 of unwinding and electrophoresis solution (right). The results represent the mean of two independent experiments $\pm$ SD value. Statistically significant differences $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ represent the difference between samples without enzymes and samples with EndoIII, FaPy or DNA inhibitors.

Discussion

In previous papers both MNNG and H_2O_2 were shown to induce DNA ssb, as detected by several biochemical methods (Slameňová et al. 1997; Gábelová et al. 1997). To ascertain the ratio of alkali-labile sites and ss DNA breaks in MNNG- and H_2O_2-treated V79 cells we assayed the samples at different pH of unwinding and electrophoresis solutions. The differences between the curves in the left part of Fig.1 show that all detected breaks in MNNG-treated cells have the character of alkali-labile sites; at pH = 12.1 there was no significant increase in the number of ss breaks. Treatment with many alkylating agents, including N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), leads to unstabilization of N-glycosyl bonds, opening of base rings, depurination/depyrimidination and the appearance of alkali-labile and apurinic/apyrimidinic (AP) sites by spontaneous hydrolysis or by the action of specific DNA glycosylases (Singer 1986). The removal of AP sites is accomplished by AP endonucleases which cleave DNA adjacent to AP sites and create ss DNA breaks.

In samples treated with 50 or 100 μmol/l H_2O_2 the level of breaks induced at pH = 12.1 was significantly lower than that at pH $\geq$ 13.0, yet the differences were small (Fig. 1 right part). We suggest that nearly all ss DNA breaks detected immedi-

ately after H_2O_2-treatment had the character of true DNA breaks and alkali-labile lesions represented only a minor type of lesions H_2O_2 crosses biological membranes, penetrates to the nucleus and reacts with Cu^{1+} and Fe^{2+} ions to form $^{\cdot}OH$ radicals. Attack of $^{\cdot}OH$ radicals on DNA leads to base modification, deoxyribose fragmentation, base loss and strand breaks (Halliwell and Aruoma 1991). Most of these DNA lesions are repaired by direct rejoining or base excision repair. Our data suggest that different types of lesions develop under different pH conditions.

Fig. 2 suggests that the effects of DNA repair enzymes and DNA inhibitors are more suitably examined under the lower pH (right part of Fig. 2), which reduces detection of alkali-labile sites in DNA. EndoIII, FaPy and DNA inhibitors increased the level of ss DNA breaks. We assume that this effect may have resulted from AP lyase activity of both DNA repair enzymes. This hypothesis will be further examined by using an enzyme whose major physiological role is an AP endonuclease activity, i. e. exonuclease III

Applications of the modified comet assay with the inclusion of enzymes specific for certain kinds of lesion might be employed in genotoxicity testing and characterisation of the damage caused by select carcinogens

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