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Detection of excision repaired DNA damage in the comet assay by using Ara-C and hydroxyurea in three different cell types

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Abstract Because of its characteristics, the comet assay has been used to evaluate the ability of virtually any type of eukaryotic cell to repair different kinds of DNA damage, including double and single strand breaks and base damage. The ability to detect excision repair sites using the alkaline version can be enhanced by the inclusion of repair inhibitors, DNA synthesis inhibitors, or chain terminators. In this sense, we evaluated the ability of hydroxyurea (HU) and cytosine arabinoside (Ara-C), for detecting lesions produced by the alkylating agents ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) in three different cell systems. Two hundred cells for experimental point were analyzed in the alkaline version of the comet assay, and the results are evidences of the utility of the assay to detect alkylation of bases in the cells lines MRC-5 and TK-6, as the treatment with HU +Ara-C significantly increases both the basal and induced frequency of DNA damage. The use of whole blood, although it detected the effects of MMS, with and without repair inhibitors, failed to detect the effect of the selected dose of EMS and does not permit detection increases in the background level.

Keywords Comet assay · Ara-C · Hydroxyurea · Alkylating agents · Human cell lines

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Introduction

Over the past decade, the comet assay or single-cell gel electrophoresis (SCGE) assay has become one of the standard methods for assessing DNA damage, with applications in genotoxicity testing, human biomonitoring, and molecular epidemiology, as well as in fundamental research on DNA damage and repair. The assay attracts adherents by its simplicity, sensitivity, versatility, speed, and economy, and the number of publications it spawns rises each year (Rojas et al. 1999; Collins 2004; Speit and Hartman 2006). Among the strengths of this protocol are its speed and simplicity. A complete assay, using several concentrations of test compound, can be conducted and scored within one working day. Scoring the assay using proprietary image analysis software is objective, and

rapid and data are immediately accessible to further analysis.

Because of its characteristics, the SCGE assay has been used to evaluate the ability of virtually any type of eukaryotic cell to repair different types of DNA damage, including double- and single-strand breaks and base damage. The neutral and alkaline versions of the assay have been used to assess the repair of double-strand and single strand breaks, respectively (Rojas et al. 1999). However, it must be recalled that many compounds are producing others lesions, not directly related with strand breaks.

Agents such as UV radiation, which produces lesions that do not form strand breaks directly, can also be evaluated by using the comet assay. In this case, rather than detecting strand breaks produced by the irradiation, it is possible to detect strand breaks produced by the attempt of the cell in repairing the lesions (Rojas et al. 1999). In addition, the ability to detect excision repair sites, using the alkaline version, can be enhanced by the inclusion of repair inhibitors, DNA synthesis inhibitors, or chain terminators (Jutras et al. 1989; Mirzayans et al. 1992; Surrallés et al. 1995; Martin et al. 1999; "" Ori et al. 2005).

The breaks that occur as intermediates in nucleotide excision repair of UV-induced damage or bulky adducts are normally short-lived, at least in proliferating cells. Incubation with DNA synthesis inhibitors like hydroxyurea (HU), cytosine arabinoside (Ara-C, 1-B-D-arabinofuranosylcytosine), or aphidicolin blocks repair patch synthesis and causes incision breaks to accumulate. This provides a sensitive way to detect the effects of damaging treatments and the spontaneous or basal DNA damage (Surrallés et al. 1995; Rojas et al. 1999; Collins 2004). Thus, although the SCGE is essentially a method for measuring DNA breaks, the introduction of lesion-specific endonucleases or repair inhibitors allow the detection of, for example, oxidized bases and alkylation damage (Collins et al. 1997a; Collins 2004). These complements introduced to the standard SCGE assay can be exploited to increase the sensitivity of the assay, to cover the detection of a wider range of genetic damage, and to detect the genotoxic potential of much more genotoxic compounds.

Hydroxyurea inhibits DNA repair by blocking DNA synthesis and affecting the replication checkpoint (Ho et al. 2006), which enhance cell cytotoxicity. The effects of Ara-C on DNA repair are visualized by its potent inhibitory effect on DNA polymerase in the long-patch excision repair process (Frankfurt 1991; Gedik and Collins 1991). The usefulness of the approach that use DNA repair inhibitors has been reported by Martin et al. (1999), showing that several of the compounds that induced micronucleus formation in MCL-5 cells, only produce increments in the comet assay in the presence of the DNA-repair inhibitors HU and Ara-C. These inhibitors allow that the recognition and incision stages of nucleotide-excision repair process occur, but inhibit the subsequent DNA resynthesis step. This results in an accumulation of single-strand breaks.

The genotoxic effects induced by alkylating agents are enhanced by the use of DNA repair inhibitors (Frankfurt et al. 1993). Thus, we have used the comet assay to detect DNA damage in cultures of human fibroblasts cells (MRC-5), human lymphoblastoid cells (TK6) and peripheral blood leukocytes from whole blood, exposed to the alkylating agents ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS), in both the presence and absence of HU and Ara-C.

Materials and methods

Chemicals

Ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS) hydroxyurea (HU), and 1-B-D-arabinofuranosylcytosine (Ara-C) were obtained from Sigma (Munich, Germany). RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin–streptomycin, amphotericin B (AMPH), low-melting point agarose (LMA), and normal-melting-point agarose (NMA) were purchased from Gibco BRL (Paisley, UK); while phosphate-buffered saline (PBS), ethylene diaminetetraacetic acid, and the stains, ethidium bromide (EtBr), trypan blue (0.4% solution), and fluorescein diacetate (FDA), were purchased from Sigma. Sodium chloride and dimethylsulfoxide (DMSO) were from Panreac Química (Barcelona, Spain); *N*-laurosylsarcosine sodium and polyethylene glycol tert-octylphenyl ether (Triton X-100) were from Fluka (Buchs, Switzerland); sodium hydroxide and absolute ethanol from Carlo Erba Reagenti (Milan, Italy); and Tris buffer was from US Biochemical (Cleveland, OH).

Cell lines

The human lung SV40 immortalized MRC5 cell line was grown in RPMI 1640 medium, supplemented with 10%

fetal calf serum, antibiotics (penicillin 90 UI/ml and streptomycin 90 μg/ml), and glutamine at 37°C.

The human lymphoblastoid TK6 cell line has a stable karyotype and has been used extensively for mutagenicity and other genotoxicity studies, including the comet assay (Honma et al. 1997; Guillament et al. 2004). TK6 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2.5 U/ml amphotericin B. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Asynchronous cultures in the exponential phase of growth were used in all experiments.

Peripheral blood samples were collected by venipuncture, using heparinized vacutainers. The whole blood was centrifuged and the supernatant removed. The cell pellet was resuspended in equal volume of RPMI 1640 medium (Gibco), and after that, each blood sample was treated.

Incubation of cells with test chemicals and DNA-repair inhibitors

Aliquots of suspensions of cells (~1×10^5 cells/75 μl) were incubated at 37°C for 30 min with graded concentrations of test chemicals in the presence or absence of the DNA repair inhibitors HU (10 mM) and Ara-C (1.8 mM). Control cells were incubated with the appropriate solvent, with or without HU and Ara-C.

Cell viability was evaluated immediately after resuspension with a mixture of FDA and EtBr (Strauss 1991). Two hundred cells were scored for each treatment. All of the treatments resulted in a minimum of 80% viable cells, a level sufficient for avoiding cytotoxicity artifacts in the comet assay (Henderson et al. 1998). Preliminary studies on the toxicity of different concentrations of EMS and MMS (Table 1) permitted to select the concentrations of 5 mM EMS and 0.5 mM MMS, as concentrations with cell viability above 80%, but inducing significant increases in DNA damage levels.

Alkaline single-cell gel electrophoresis

The comet assay was performed under alkaline conditions, according to the method of Singh et al. (1988), with some minor modifications. Slides previously kept in ethanol–ether (2:1), and after, in 70% ethanol at 4°C, were used. Two solutions, one containing 0.5% normal melting agarose (NMA) and the other containing 0.5% low melting agarose (LMA) were prepared. NMA, 150 μl, were transferred onto the slide, evenly spread, and kept at 60°C for 10 min to solidify the agarose. Of 0.5% LMA, 75 μl were mixed with 15 μl of the cell suspensions or 7.5 μl of peripheral blood and layered onto the slides, which were then immediately covered with coverslips. After agarose solidification at 4°C for 10 min, the coverslips were removed and the slides were immersed overnight at 4°C in freshly lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl) containing 1% Triton X-100 and 10% dimethylsulfoxide, added just before use. The slides were equilibrated in alkaline solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) for 20 min. Electrophoresis was carried out for 20 min at 25 V and 300 mA. Alkali treatments and electrophoresis were performed in an ice bath. After this, slides were neutralized by washing two times with 0.4 M Tris–HCl buffer (pH 7.5) every 5 min and 100% ethanol. Slides were stained with 60 μl of ethidium bromide solution in distilled water (0.2 mg/ml) (dilution:1/250). One hundred randomly selected comet images were analyzed per treatment. Each experimental point was scored by duplicate. Thus, two hundred comets were analyzed per treatment.

Observations were made at ×400 magnification using a fluorescent microscope (Olympus BX50, equipped with a 515–560 nm excitation filter) connected through a Hitachi Denshi, Ltd. CCD-IRIS Color Video Camera. The image for each individual cell was acquired immediately after opening the microscope shutter to the computer monitor, employing the Komet 5.5 Program. Cells were analyzed

Table 1 Results from a preliminary study on the viability of EMS and MMS treatments

Compound	15 mM	10 mM	5 mM	1 mM	0.5 mM	0.25 mM	control
EMS	96	95	97	97	–	–	97
MMS (1)	–	82	81	80	80	–	82
MMS (2)	–	–	–	96	96	97	97

The study was carried out in the TK-6 cell line. Viability is indicated in percentages. (1) and (2) correspond to two different experiments.

according to their Olive Tail Moment (OTM) and the percentage of DNA in tail.

Statistical analysis

The Kolmogorov–Smirnov test was carried out for each sample. A result was considered statistically significant when $P \leq 0.05$. Data were analyzed by means of the Mann–Whitney and *t* Student tests.

Results

Preliminary studies indicated that MMS is much more toxic than EMS, and as consequence, in this experiment, the selected concentration of MMS was tenfold lower than the concentration of EMS. Thus, in our experiments, 5 mM EMS induced 94% viability, when tested without inhibitors, and 96%, when inhibitors were used. Experiments with 0.5 mM MMS show viabilities of 96%, when evaluated without inhibitors, and 95%, when inhibitors were included. These results confirm that concentrations were well selected. In addition, the concentrations selected for the two inhibitors were 10 mM for HU and 1.8 mM for Ara-C. As it occurs with the selected alkylating agents, the doses selected of the inhibitors did not induce significant reductions in cell viability.

Results obtained with the MRC-5 cell line are indicated in Table 1. The results indicate that the treatments with the two inhibitors alone, detect a low increase in the average of the OTM and in the percentage of DNA in tail in the control experiment. As expected, the two alkylating agents induce clear and significant increases in the frequency of DNA damage, the effects induced by MMS being higher than those induced by EMS. It is observed that the co-treatments with the inhibitors induce significant increases in the average of DNA damage, over the levels induced by the alkylating agents alone. The detection of damage repaired by NER is clearly observed after treatment with MMS, as increases close to 80% of the induced by MMS alone were observed. It must be indicated that the observed effects were clearly significant ($p<0.001$) (*t* test and Mann–Whitney test) for both treatments and co-treatments.

The data obtained with the TK-6 cell line are indicated in Table 2. The observed results are quite similar to that obtained with the MRC-5 cell line: slight increases observed by the basal NER repair in control, and higher genotoxicity of MMS with respect to EMS. The effect of the NER repair is clearly significant when challenged with the two alkylating agents; nevertheless, in this case, the induction of NER-repairable damage induced by EMS is proportionally higher than with MMS. Thus, increases of 107% are observed after co-treatment of MMS and the repair inhibitors used. As with the MRC-5 cells, the effects observed in the TK-6 cell line were clearly significant ($p<0.001$) (*t* test and Mann–Whitney test) for both treatments and co-treatments.

Table 2 DNA damage induced by EMS and MMS, with and without repair inhibitors

Treatment	Olive tail moment Mean ± SD	Percent DNA in tail (%) Mean ± SD
Control	1.25±0.10	10.77±0.76
Control/ HU + Ara-C	1.52±0.11	12.55±0.72
EMS	3.29±0.24[a]	20.46±1.08[a]
EMS/ HU + Ara-C	4.65±0.18[b]	25. 61±0.90[b]
MMS	9.76±0.31[a]	57.94±0.80[a]
MMS/ HU + Ara-C	17.26±0.51[b]	64.08±0.93[b]

Results obtained in the MRC-5 cell line.

[a] Significantly different with respect to the control

[b] Significantly different with respect to the treatment with the alkylating agent alone

Results obtained with samples of whole blood are slightly different than the ones reported above, as EMS, at the concentration tested, was not able to induce significant increases on the levels of DNA damage (Table 3). The co-treatment with the repair inhibitors did not induce increases in the background levels of DNA damage in blood leukocytes, and the same is observed after the treatment with the alkylating agent EMS. This experiment was repeated twice and the results were quite similar. This would indicate any kind of interactions between some of the blood components and the repair inhibitors. Nevertheless, MMS induce clear and significant levels of DNA damage that are increased with the repair inhibitors treatment.

It should be indicated that although the two selected parameters, OTM and % DNA in tail, follow the same tendency, the increases observed when the OTM is used are slightly higher than when the % DNA in tail is used.

The overall results obtained in this study are summarized in Fig. 1.

Table 3 DNA damage induced by EMS and MMS with and without repair inhibitors

Treatment	Olive tail moment Mean ± SD	Percent DNA in tail (%) Mean ± SD
Control	2.10±0.17	16.18±1.04
Control/ HU + Ara-C	2.98±0.22	19. 21±0.99
EMS	4.18±0.19[a]	24.63±0.87[a]
EMS/ HU + Ara-C	8.65±0.37[b]	37.47±1.10[b]
MMS	9.49±0.29[a]	40.77±0.92[a]
MMS/ HU + Ara-C	15.16±0.41[b]	58.75±0.88[b]

Results obtained in the TK-6 cell line

[a] Significantly different with respect to the control

[b] Significantly different with respect to the treatment with the alkylating agent alone.

Discussion

The single cell gel electrophoresis (SCGE) or comet assay is a rapid and sensitive fluorescent microscopic method that allows measurement of DNA strand breaks in individual cells (Singh 2000). In its alkaline version, it detects DNA damage as single-stranded and/or double-stranded DNA breaks at the level of eukaryotic genome, as well as alkali-labile sites, incomplete DNA repair sites, and changes in structural chromosome configuration (Tice et al. 2000).

As it occurs with other many genotoxicity assays, modifications of the SCGE standard protocol permit to detect different specific types of DNA damage. These modifications obviously improve the significance of the assay as it permits to classify as genotoxic, compounds that usually give negative results in the standard assay. In addition, these modifications also permit to determine the different mechanism of action inducing DNA damage. The use of FPG and Endo III enzymes are often employed as a complement of the comet assay. The use of such enzymes permit to detect purine and pyrimidine oxidized bases, which are frequent DNA modifications generated both spontaneously and induced by many genotoxic agents (Collins et al. 1993). In addition to the detection of oxidative damage, the comet assay also provides a useful system for assessing the repair activity of cells. Thus, extensions of the standard method using repair enzymes or repair inhibitors add greatly to the potential applications of the assay (Collins et al. 1997b).

Alkylated DNA bases is another very common lesion playing an important role in cancer induction. To detect such lesions, the enzyme 3-methyladenine-DNA glycosilase II (AlkA) has been used (Collins et al. 2001; Blasiak et al. 2004). This enzyme has a broad substrate specificity including alkylated purines (7-methylguanine) and 3-methyl adenine, and O^2-alkyl pyrimidine and hipoxanthine (Laval 1977; Tudek et al. 1998).

It is known that alkylated bases initiate several excision repair mechanisms, which involve incision of the DNA strand, excision of the damaged nucleotide, gap filling by

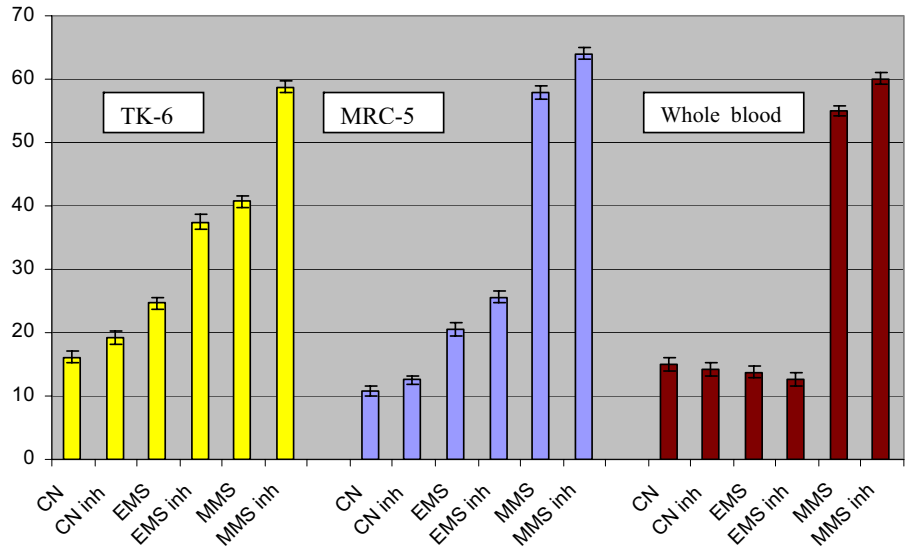

Fig. 1 Summary of the results obtained in MRC-5, TK-6, and whole blood cells by EMS and MMS with and without repair inhibitors

DNA resynthesis, and rejoining by ligation (Yamauchi et al. 2002). Thus, another way to detect the overall effects on DNA damage induced by alkylating agents is by using DNA repair inhibitors interfering the repair mechanism that eliminated DNA alkylations. Two of these repair inhibitors are HU and Ara-C, which interfere in the excision repair process. When testing the genotoxic damage of a compound in the presence of such inhibitors, in addition to the breaks directly induced by these agents, single-strand breaks induced by avoiding the gap filling during NER process are also measured (Horvathova et al. 1999). These repair inhibitors are known to sensitize murine and human cells to a variety of mutagens including radiation and radiomimetic compounds (Preston 1980; Fram and Kufe 1985). The inhibition induced by Ara-C may be due to either direct inhibition of the DNA polymerase when AraCTP is bound to the dCTP binding site of the enzyme or to indirect inhibition through AraCMP incorporation into a repairing region of DNA rendering unsuitable for further polymerase action (Major et al. 1982). This function is dependent of the p53 status of the cell, as no increases in the frequency of chromosomal damage in mouse embryo fibroblasts exposed to bleomycin were observed in a p53 −/− background (Allio et al. 2000). On the other hand, HU prevents DNA replication by selectively inhibiting ribonucleotide reductase with S-phase cell-cycle specificity and some potential to arrest cells at the G1/S boundary (Frias et al. 1996). It has been shown that HU, in combination with Ara-C neither causes DNA breaks nor affected cell survival during the first 10 h (Filatov et al. 1998). This combination is often used in both in vitro and in vivo studies. In in vitro studies, it has been useful to demonstrate the genotoxicity of pesticides in human lymphocytes (Williams et al. 2004) and in several model compounds in a metabolically competent lymphoblastoid cell line (Martin et al. 1999).

Measuring DNA strand breaks gives limited information on how an agent interacts with DNA. Breaks may represent the direct effect of some damaging agent, but they are generally quickly rejoined. In addition, they may also be intermediates generated in cellular repair process because both nucleotide and base excision-repair processes cut out damaged bases and replace them with sound nucleotides. Thus, in our study, we have tried to corroborate that the comet assay is more specific and more sensitive, when the standard protocol is modified by introducing an extra step using repair inhibitors. Two standard alkylating model agents (EMS and MMS) have been selected, and their effects have been tested in three different cell line models, as the repair efficiency may be affected by the makeup repair background of the selected cells.

It this exploratory survey, the two selected compounds were tested in different cell lines, using the standard protocol in the absence or presence of HU + Ara-C. Treatments last for short exposure periods (30 min).

The slight but statistically significant effect of HU + Ara-C on comet formation in control cells, as demonstrated in the present study, suggests the presence of low levels of endogenous DNA alkylation damage, as it has been already demonstrated (Ori et al. 2005). The treatment with both alkylating agents produced significant increases in the comet parameters used to measure DNA damage, and this was observed in the two cell lines in the absence of HU + Ara-C. Nevertheless, in the presence of the repair inhibitors, this damage was markedly increased, which emphasize the usefulness of the use of this combination of repair inhibitors. This behavior was a bit different when whole peripheral blood was used. In this case, negative results were obtained with EMS (Table 4). No increases were observed either with the EMS alone or with the addition of repair inhibitors, and the same occurs at the background level (control). It is possible that this lack of activity may be related with some interactions between blood components and the repair inhibitors, as well as with EMS.

In peripheral lymphocytes, it has been reported that repair incision breaks accumulate without inhibitors, as the

Table 4 DNA damage induced by EMS and MMS with and without repair inhibitors

Treatment	Olive tail moment Mean ± SE	Percent DNA in tail (%) Mean ± SE
Control	2.98±0.30	14.97±1.02
Control/ HU + Ara-C	2.80±0.32	14.15±1.12
EMS	2.69±0.24	13.78±0.99
EMS/ HU + Ara-C	2.40±0.23	12.64±0.96
MMS	13.68±0.31[a]	54.94±0.79[a]
MMS/ HU + Ara-C	17.31±0.65[b]	64.03±0.90[b]

Results obtained in human peripheral blood cells

[a] Significantly with different respect to the control

[b] Significantly with different respect to the treatment with the alkylating agent alone

rate of religation is limited by the poor supply of deoxyribonucleoside triphosphates (Collins 2004). However, in our case, the spontaneous level of DNA damage observed in blood cells is intermediate to the observed in the two established cell lines. On the other hand, it must be indicated that the response to genotoxic treatments may be cell line dependent, as demonstrated in other studies (Little et al. 1989).

Some authors have used only Arac-C as repair inhibitor to detect basal and induced levels of DNA damage. In a biomonitoring study carried out with traffic warders, although the use of Ara-C do not permit to detect the effect of the exposure, increases in the background DNA damage were detected that permits to detect the effect of age on the frequency of micronuclei (Leopardi et al. 2003); this prove the usefulness of this approach inclusive in in vivo studies. In addition, the addition of HU (to Ara-C) enhanced the sensitivity of the micronucleus assay in response to certain genotoxins (Martin et al. 1999). Thus, the usefulness of using the combination HU + Ara-C, as it has been demonstrated in our work with alkylated DNA damage, may be extended to all those exposures inducing any kind of DNA damage repairable by the excision repair mechanisms.

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