Differentiated and exponentially growing HL60 cells exhibit different sensitivity to some genotoxic agents in the comet assay

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ABSTRACT

The aim of this study was to investigate the effect of the cell differentiation status on the sensitivity to genotoxic insults. For this, we utilized the comet assay to test the DNA damage after treatment with 5 different substances with different mechanism of action in human promyelocytic HL60 cells with or without cell differentiation.

A 4-hour MMS treatment induced a significant and concentration-dependent increase in DNA damage for both differentiated and undifferentiated cells, but the difference in sensitivity was only significant at the highest concentration. A 4-hour doxorubicin treatment did not induce DNA damage in differentiated HL60 cells, while it did in undifferentiated cells with its highest tested concentration. A one-hour etoposide treatment caused significant increase in DNA damage concentration dependently in both cell variants. This DNA damage was significantly higher in undifferentiated HL60 cells with several tested concentrations of etoposide. The treatment with the oxidizing substances hydrogen peroxide and potassium bromate yielded significant DNA damage induction in both undifferentiated and differentiated cells with no difference according to the differentiation status.

Doxorubicin and etoposide are known to inhibit topoisomerase II. The activity of this enzyme has been shown to be higher in undifferentiated actively proliferating cells than in differentiated cells. This may be of relevance when exposures to topoisomerase-inhibiting compounds or the genotoxicity of compounds with unknown mechanism of action are assessed in routine testing.

1. Introduction

The promyelocytic cell line HL60 was established from a female patient with acute myeloid leukaemia \cite{1}. The cells display a myeloblastic/promyelocytic morphology and carry typical surface antigens and some other characteristics of immature myeloid cells \cite{2}. With this, they represent a maturation state that is between pluripotent hematopoietic and myeloid stem cell. Since hematopoietic cancers induced by chemical agents originate in less differentiated cell stages during the maturation of hematopoietic stem cells, and because about 25 of the 100 known human carcinogens induce leukaemia or lymphomas \cite{3}, HL60 cells may be considered a suitable model for the investigation of mutagenic chemicals.

HL60 cells can differentiate into a granulocyte-like or a monocyte-like form. This occurs spontaneously during cell culture and about 10\% of cells are differentiated during normal culture \cite{2}. Upon treatment with certain chemicals, differentiation of the majority of the cultured cells (up to 90\%) can be induced \cite{2}. For example, with DMSO or retinoic acid differentiation is into granulocyte-like cells and with phorbol esters or sodium butyrate cells differentiate into a monocytelike cell type \cite{2}. This enables the investigation of the relevance of the differentiation state for the sensitivity to genotoxic agents. However, only tests that do not require cell proliferation or mitosis can be applied, because differentiated cells do not divide any more. The comet assay fulfils this condition as it is suitable for any cell type from which a single cell suspension can be prepared.

While the majority of in vitro genotoxicity testing is performed with actively dividing cultured cells, there are some circumstances in which genotoxicity is routinely assessed in differentiated cells. In rodents, the comet assay is used for the assessment of organ-specific genotoxic effects in the context of mutagenicity testing during the registration process of substances \cite{4}. Most of these organ-derived primary cells are
not in a state of exponential growth. In human biomonitoring, peripheral mononuclear cells are used, which is a population consisting of cells in different differentiation stages. The comet assay has also been employed in neurotoxicity testing for the measurement of DNA damage in differentiated brain cells [5,6].

For HL60, certain differences like level of cellular glutathione and topoisomerase II between the undifferentiated and the differentiated state have been described (e.g. [2,7–9]), but so far there is no published genotoxicity study comparing both states for their sensitivity.

We tested 5 compounds with various mechanisms of action for which the comet assay shows good sensitivity. Hydrogen peroxide (H₂O₂) and potassium bromate (KBrO₃) are known to induce oxidative stress, methyl methane sulfonate (MMS) is an alkylating agent, doxorubicin is an intercalating substance and etoposide a topoisomerase II inhibitor. Differentiated and undifferentiated cells were treated in parallel and were subjected to electrophoresis on two gels of the same slide, minimizing intra- and inter-experimental variation.

2. Materials and methods

2.1. Materials

Unless stated otherwise, chemicals were purchased from Sigma Aldrich Germany (Munich, Germany). Cell culture media and reagents were purchased from PAA Laboratories (Passing, Austria) and Life Technologies (Darmstadt, Germany). Fetal bovine serum (FBS) was from Biochrom (Berlin, Germany). Normal melting point agarose and super frost slides were from Carl Roth (Karlsruhe, Germany). The GelGreen nucleic acid stain was purchased from Biotium (Hayward, CA, USA). Dimethyl sulfoxide (DMSO) was obtained from Carl-Roth (Karlsruhe, Germany). HL60 cells were obtained from R. Schinzel, Vasopharm (Wuerzburg, Germany).

FPG protein (Formamidopyrimidine-DNA glycosylase of Escherichia coli) was kindly supplied by Prof. Dr. Bernd Epe (University of Mainz).

2.2. Methods

2.2.1. Cell culture

HL60 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, 1% (w/v) l-glutamine and 0.4% (w/v) antibiotic (penicillin/streptomycin) in an incubator with 5% CO₂ at 37 °C. The differentiation of HL60 cells was achieved with 1.25% DMSO treatment over 72h, conditions which had been found optimal in preliminary experiments. The culture medium supplied with 1.25% DMSO was renewed every 24 h. After 48 h of differentiation, the cell number of differentiated HL60 was scored in order to seed undifferentiated HL60 cells at an adjusted density of half of that of the differentiated cells. This provided equal cell densities for both cell types at the time of substance treatment. After 72 h of differentiation, differentiated and undifferentiated (seeded one day prior to the treatment) HL60 cells were treated with the test substances (4 h with methyl methane sulfonate (MMS; 25, 50, 75, 100, 150 and 250 μM) and doxorubicin (50, 100, 150, 200, 250 and 500 μM); one hour with etoposide (0.1, 0.5, 1, 5, 10 and 25 μM); 30 min with hydrogen peroxide (H₂O₂; 25, 50, 75, 100, 125, 150 and 175 μM) and 3 h with potassium bromate (KBrO₃; 50, 100 and 150 μM)).

2.2.2. Morphological analysis of differentiation status

The differentiation status of the HL60 cells was determined morphologically according to nuclear morphology (granulocyte-like shape, see Fig. 1). In previous experiments, it had been confirmed that this appearance correlates with an increase of the differentiation-specific surface marker CD11b [10]. We had also confirmed that the expression of the insulin receptor is markedly decreased in this state as described in the literature [11] (and own unpublished observation). At the end of the 72 h, the cells (differentiated and undifferentiated) were placed on a glass slide by cytospin centrifugation (1000 rpm for 5 min, 30,000 cells/slide). The slides were fixed in cold methanol (−20 °C) for at least 2 h and stained with GelGreen nucleic stain (1:100 diluted in bidistilled water). Coverslips were mounted with Dabco and 400-cells per sample (2 × 200 cells per group) were analysed with an Eclipse 55i microscope (Nikon GmbH, Düsseldorf, Germany) at 400-fold magnification by using a FITC filter to ensure a differentiation of more than 80% of cells.

2.2.2. Comet assay

After treatment of the cells with test substances (see 2.2.1), cells were harvested and 20 μl of cell suspension (contains 3500 cells/μl) was mixed with 180 μl of 0.5% low melting point agarose at 37 °C. Thirty-eight μl of cell-agarose mixture was placed on a fully frosted slide that was coated with 1.5% of normal melting point agarose (University of Mainz). The slides were placed in an electrophoresis chamber that was filled with fresh electrophoresis buffer (300 mM NaOH and 1 mM Na₂EDTA, pH 13) for 30 min at 4 °C in dark (20 min for FPG comet assay). Then the electrophoresis was performed for 30 min (20 min for FPG comet assay) at 25 V (1.1 V/cm). To neutralize the slides, they were immersed in 0.4 M Tris buffer (pH 7.5) for 5 min and then to dehydrate in methanol for 5 min at ~20 °C. For FPG comet assay, an enzyme treatment step was performed directly after the lysis step and before the alkaline unwinding. For each treatment group two agarose gels were treated with FPG enzyme (8 μg/ml protein, 100 μl per gel) and two agarose gel replicates from the same sample were treated with the enzyme buffer (40 mM Hepes, 100 mM KCl, 0.5 mM Na₂EDTA and 0.2 mg/ml BSA, pH 8.0). The treatment was at 37 °C for 30 min. After the enzyme treatment, the slides were washed with enzyme buffer and

![](image-url)
the rest of the steps after the lysis were performed as described above (alkaline unwinding for 20 min and electrophoresis for 20 min). After the methanol fixation, the slides were left to dry under a fume hood and stained with 20 μl of Gel red/Dabco solution. Evaluations of the slides were done with a fluorescence microscope (Labophot 2; Nikon) at 200-fold magnification and image analysis software (Komet 5, BFI Optilas, Germany). One hundred randomly selected cells (50 per replicate agarose gels) for each treatment were analysed. The percentage of DNA in the tail was used to quantify DNA damage. The FPG comet assay results are presented as Net DNA in tail, which was calculated as the percent of DNA in tail with FPG after subtraction of the values without FPG according to following formula.

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\text{Net DNA in tail} \% = \frac{\text{DNA in tail (\% FPG) - DNA in tail (\% w/o FPG)}}{\text{DNA in tail (\% FPG)}}
\]

2.2.4. Viability test
At the time of the cell harvest for comet assay, a cell viability test was performed with the same cell pellet. For this, 35 μl of cell suspension were mixed with 15 μl of staining solution (2 μl Gel Red stock solution and 12 μl fluorescein diacetate (FDA; 5 mg/ml in acetone) in 2 ml PBS) and 45 μl of this mixture was applied on the slide and covered with a cover slip. FDA is activated to exhibit green fluorescence by cytosolic esterases in intact cells, while Gel Red can only enter cells with compromised membrane integrity. In total 200 cells (red and green stained) were scored with an Eclipse 55i microscope (Nikon GmbH, Düsseldorf, Germany) at 200-fold magnification by using FITC filter. The proportion of vital cells to dead cells was determined.

2.2.5. Statistics
Statistical analysis was performed by using SPSS 22 software. The data are presented as Mean ± SD. The Shapiro-Wilk test was performed to test the normality of the data and the Mann-Whitney-U test was used to compare the differences between two treatment groups. The results with a p value ≤0.05 were considered as significant.

3. Results

3.1. Differentiation status of HL60 cells after 72-h-DMSO treatment
The differentiation status of the HL60 cells following to 72 h DMSO treatment was assessed microscopically. Clear morphological alterations of the shape of the cell nucleus were observed in the DMSO treated group (Fig. 1). With a 72-h-DMSO treatment, 89.40 ± 5.30 (Mean ± SD) percent of the HL60 population were differentiated (n = 40; average of all controls within the time frame of the experimental work for this publication). In regular HL60 culture (without DMSO treatment), 15.01 ± 4.13 (Mean ± SD) percent of the HL60 population was spontaneously differentiated (n = 40).

3.2. DNA damage induction by methyl methanesulfonate (MMS) treatment
Comet assay analysis revealed that a 4-hour MMS treatment yielded a concentration-dependent increase in DNA damage which was significant with 100 μM or more in undifferentiated and differentiated HL60 cells. Differentiated HL60 cells showed slightly lower DNA damage compared to the undifferentiated cells. However, this difference between undifferentiated and differentiated cells only became significant with the highest tested concentration of MMS (250 μM) (Fig. 2) and in another set of experiments with 150 μM MMS, when it was used as positive control in the doxorubicin treatment (Fig. 3).

3.3. DNA damage induction by doxorubicin treatment
A 4-hour doxorubicin treatment led to an increase in DNA damage in undifferentiated HL60 cells in the comet assay, which was significant with 500 nM. In contrast, differentiated HL60 cells did not show any increase in DNA damage with doxorubicin treatment. In this experiment, 150 μM of MMS treatment was added as positive control, which caused a significant increase in DNA damage in both cell types. Thus, a significant difference in DNA damage levels induced by doxorubicin was observed between undifferentiated and differentiated HL60 cells with the undifferentiated cells reacting with higher sensitivity (Fig. 3).

3.4. DNA damage induction by etoposide treatment
An hour etoposide treatment yielded a concentration dependent increase in DNA damage for undifferentiated and differentiated HL60 cells in the comet assay. The lowest concentration, which caused a significant increase of DNA damage was 0.5 μM for undifferentiated HL60 cells and 1 μM for differentiated HL60 cells. The difference between undifferentiated and differentiated cells in comet assay was significant with 0.5 μM etoposide and higher concentrations. The undifferentiated HL60 cells reacted to the etoposide treatment with higher sensitivity than the differentiated cells (Fig. 4).

3.5. DNA damage induction by hydrogen peroxide treatment
A 30 min hydrogen peroxide (H₂O₂) treatment led to a significant increase in DNA damage with doxorubicin treatment. In this experiment, 150 μM of MMS treatment was added as positive control, which caused a significant increase in DNA damage in both cell types. Thus, a significant difference in DNA damage levels induced by doxorubicin was observed between undifferentiated and differentiated HL60 cells with the undifferentiated cells reacting with higher sensitivity (Fig. 3).
increase of DNA damage with all used concentrations in both cell types. An increasing amount of DNA damage was observed with increasing H2O2 concentrations. The differentiation status of the HL60 cells did not show any effect on DNA damage after this treatment (Fig. 5). A FPG comet assay was utilized to determine the differences specifically in oxidative DNA damage after hydrogen peroxide treatment (25, 50 and 75 μM). A concentration dependent increase was observed after FPG enzyme treatment in both cell types (undifferentiated and differentiated HL60). However, there was no difference in induced oxidative DNA damage between the undifferentiated and differentiated cells (Fig. 6).

3.6. DNA damage induction by potassium bromate treatment

A 3-hour potassium bromate (KBrO3) treatment did not cause DNA damage induction without FPG treatment (not shown). After the incubation with FPG enzyme, a concentration dependent increase in DNA damage was observed for both cell types (undifferentiated and differentiated HL60). All chosen concentrations of potassium bromate yielded a significant increase in DNA damage after the FPG-incubation. There was no difference in DNA damage levels between undifferentiated and differentiated cells (Fig. 7).

3.7. Cell viability

The tested substances did not induce cytotoxic effects in undifferentiated or differentiated HL60 cells after the applied treatment periods (4 h for MMS and doxorubicin, one hour for etoposide, 30 min for H2O2 and 3 h for KBrO3) and the applied conditions; there was no significant difference to the respective solvent controls (data not shown).

4. Discussion

HL60 cells were induced to differentiate into a granulocyte-like cell type by treatment with DMSO over 72 h. The differentiated state was compared with exponentially growing undifferentiated HL60 cells within one experiment in parallel treatments and in two gels on the same slide during comet assay procedures. The treatment conditions for the different genotoxic agents were chosen according to prior experiences. A 4-hour treatment duration was performed for MMS and doxorubicin, while etoposide treatment duration was one hour. A 30 min
treatment was chosen for H$_2$O$_2$ and a 3-hour treatment for KBrO$_3$. None of the test substances induced cytotoxicity under the applied treatment conditions.

MMS treatment yielded a slight difference in DNA damage between undifferentiated and differentiated HL60 cells only at the highest tested concentration. However, a difference in sensitivity between the cell types was much more apparent after doxorubicin treatment, for which the differentiated HL60 cells were insensitive. This is in accordance with a report by Ganapathi et al. [12], in which a doxorubicin resistance of HL60 cells was detected after differentiation with retinoic acid. Two main mechanisms of action can be named for doxorubicin: DNA intercalation leading to topoisomerase II inhibition and reactive oxygen species formation. To determine which mechanism causes the difference in sensitivity, we applied the specific topoisomerase II inhibitor etoposide next. The undifferentiated HL60 cells showed significantly higher DNA damage against etoposide treatment compared to the differentiated HL60 cells. To test whether the reactive oxygen species are also a contributing factor to the different sensitivity, we then applied H$_2$O$_2$ and KBrO$_3$ (with FPG). Both compounds induced very similar amounts of damage in both cell types. In line with this, in a test for oxidative stress production, flow cytometry measurement of oxidation of the dye DCF-DA, doxorubicin did not induce an effect in differentiated or undifferentiated HL60 cells (data not shown). Thus, it seems that topoisomerase II inhibition may be a mechanism for which differentiated cells are less sensitive. Actually, different amounts of topoisomerase II expression after differentiation (myeloid differentiation, granulocytic) had been described in HL60 cells earlier [7]. Topoisomerase II alpha (topo II α), the isoform which is involved in the preparation of chromatin for mitosis, decreases during differentiation of HL-60 cells, while topoisomerase II beta (topo II β) expression remains largely unchanged. Etoposide can inhibit both isoforms of topoisomerase II, but shows more affinity to the II alpha isoform [13–15].

Here, we did not observe different sensitivities for reactive oxygen species, as shown by H$_2$O$_2$ and KBrO$_3$ treatment. However, Covacci et al., [5], demonstrated higher ROS levels and higher DNA damage in undifferentiated HL60 cells compared to the DMSO-differentiated HL60 cells after H$_2$O$_2$ treatment. They explained this by an increased glutathione level after cell differentiation. This difference with our investigation might be due to the variations in HL60 cells. It is known that after the initial culture sub-lineages with slightly varying genome have developed over the last decades [16].

Another potential difference between differentiated and undifferentiated cell states might be the DNA repair capacity. Differentiated cells do not replicate any more, and therefore do not transmit genomic damage to daughter cells. Since only functionality of expressed proteins has to be ensured after differentiation, the removal of DNA lesions might be less stringent. Lee and colleagues [17] investigated the effect of differentiation status on expression and activity of human 8-oxo-G-DNA glycosylase 1 (hOGG1). They showed increased expression level of OGG1 both after granulocytic (by DMSO treatment) and monocytic (by vitamin D3 treatment) cellular differentiation. However, these findings did not show any correlation with the cleavage activity of hOGG1 enzyme. Opposite to expression, the cleavage activity of hOGG1 was reduced after granulocytic differentiation in HL60 cells. In addition, nucleotide excision repair (NER) of UV-induced lesions has been found less efficient in TPA-differentiated HL60 cells (monocyte/macrophage differentiation) [9]. Hsu and colleagues [18] showed a reduced repair of cisplatin induced crosslinks after differentiation of HL60 cells (monocyte/macrophage differentiation). None of the substances tested here relies solely on NER for the induction of DNA damage in the comet assay, but NER might play a role in the MMS-induced damage which may explain the difference observed between undifferentiated and differentiated cells at the highest tested concentration (higher % DNA in tail due to higher incision activity in undifferentiated cells). Therefore, it would be interesting to investigate NER activity further for differentiation of HL60 into the granulocyte-like cell type.

The question whether the observed differences in sensitivity due to differentiation also occur in other cell types is still open. We used the human colon cancer cell line Caco2 cells as an alternative cellular system, in which differentiation is induced by contact inhibition after 21-day continued culture. At this time the cells form a confluent monolayer and show a typical formation of cell domes. The undifferentiated Caco2 cells showed significantly higher DNA damage after one-hour etoposide treatment compared to the differentiated Caco2 cells [19], which may support the idea that not only HL60 cells, but many/most or all cells reduce the expression of topo II α during differentiation. In an earlier study [20], we had observed reduced induction of damage in the comet assay with the topo II inhibitors etoposide and amsacrine during the course of in vitro induced differentiation of the mouse embryonal carcinoma cell line F9. These ideas are also in line with observations by Prosperi et al. [21], that resting peripheral lymphocytes express very low levels of topo II α and β, but still 2.5 fold more topo II β than topo II α; upon PHA-stimulation, topo II α and β levels increased up to 30-fold (α) and 10-fold (β) to before. When Gajski et al. [22], quantified the response of peripheral lymphocytes to etoposide, they found a 10-fold higher sensitivity in the micronucleus test which requires PHA-stimulation than in the comet assay, which only yielded a significant response after a 48-hour treatment with 10 μM etoposide in resting lymphocytes. Furthermore, Kersting et al. [23], reported that the different response regarding apoptosis of peripheral lymphocytes to doxorubicin between individuals depended on the activity of topo II β, which varied 16-fold between individuals. In a comparison between proliferation-stimulated human hematopoietic stem cells (HSC) and the human lymphoblast immortalized cell line TK6 we observed a lower sensitivity in HSC to micronucleus induction and apoptosis after treatment with MMS or doxorubicin [24]. These two cell types may represent different stages of cell differentiation within the human hematopoietic cell system, but were both proliferating at the time of assessment in the micronucleus test. Since stem cells are considered a target for carcinogenesis, a comparison of resting stem cells with proliferating stem cells would be a very important future study.

Overall, our findings show that the cell differentiation status can have an impact on the sensitivity of cell systems against certain genotoxic insults. This should be kept in mind in mutagenicity testing of uncharacterized substances as well as in biomonitoring of human exposures.

Declaration of interest

None.

Acknowledgments

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