A short low-level exposure to metavanadate during a cell cycle-specific interval of time is sufficient to permanently derange the differentiative properties of Mel cells

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Mouse erythroleukemia (Mel) cells have a cell cycle-dependent high sensitivity to chemical and physical mutagens. This report shows that a 5 h exposure to 0.1 or 0.01 µg/ml metavanadate during the initial period of erythroid differentiation induction was sufficient to permanently damage the ability of treated Mel cells and their progeny to undergo erythroid differentiation, without affecting cell viability and proliferation. Conversely, a 5 h pulse of metavanadate at 1 or 10 µg/ml inhibited both differentiation and cell proliferation. The cell cycle-dependent period of mutagenesis was essential for fixation of damage in the cell genome and the progeny of the cells treated with 0.1 or 0.01 µg/ml metavanadate stably inherited an impaired capacity to differentiate. The efficiency of the DNA repair synthesis machinery during the specific period of exposure of Mel cells seemed directly involved in damage fixation. In fact, the mutagenic effects of a 0.1 µg/ml metavanadate pulse was further increased in the presence of 1 mM hydroxyurea, an inhibitor of DNA repair synthesis. In contrast, 5 µg/ml vanillin, an antimutagenic agent that stimulates repair, completely restored the capacity of progeny of cells treated with 0.1 µg/ml metavanadate to complete differentiation. Determination of [3H]deoxythymidine in acid-insoluble DNA indicated that incorporation was stimulated by metavanadate alone and was further increased by metavanadate plus vanillin; conversely, incorporation of thymidine was reduced in the presence of hydroxyurea. The capacity of metavanadate to permanently damage Mel cell erythroid differentiation appeared to depend on the cell cycle-related efficiency of the DNA repair systems, activated to correct the induced alteration, rather than on a specific concentration.

Introduction

Recently attention has been focused on the assessment of biological risks of low-level exposure to environmental pollutants. The potential risk of mutagens at low doses has commonly involved a downward extrapolation from the observed dose–response effect (Davis and Farland, 1998), however, the necessity of a new generation of biologically based dose–response models for low-level exposures to genotoxic chemicals has been proposed (Lovell, 2000). Potential risks can derive from inaccurate evaluation of the effects of low-level exposures to a compound which has a well-known mutagenic effect at higher doses (Gaylor et al., 1998). Moreover, the activation of DNA repair, to restore functions damaged by low doses of a mutagen, does not exclude the possibility that the DNA region is still modified.

We have previously studied the determination of the mutagenic potential of compounds at dose levels well below the cytostatic and cytotoxic concentrations, using mouse erythroleukemia (Mel) cells as a model system (Foresti et al., 1990, 1993). Mel cells are a permanent cell line blocked, by viral transformation, at the proerythroblastic stage of the erythroid differentiative pathway. As proerythroblasts these cells proliferate continuously. If an inducer is added to their culture medium Mel cells are switched to progress to erythropoiesis and, after 4–5 days culture, show many of the characteristics of the erythrocyte, such as hemoglobin (Hb) accumulation and growth arrest (Friend et al., 1971). This in vitro inducible hemopoietic cell line is useful for studies of mutagenesis, as the effects of mutagens can be evaluated not only on a specific cell type, the proerythroblast, but also on the process of differentiation to a different cell type, the erythrocyte. The occurrence of two distinct periods of high sensitivity to mutagens during the cell cycle of differentiating Mel cells has permitted us to study the mutagenic effects of a compound at doses not altering cell viability and proliferation (Foresti et al., 1997). There is evidence that this sensitivity is probably caused by local decreased efficiency of the DNA repair machinery during the selected period of exposure to the mutagen (Foresti et al., 1993).

Vanadum is a metal compound that is at present abundant on the Earth’s crust and is released into the environment in the form of metal oxides through the combustion of fossil fuels for energy production (Owusu-Yaw et al., 1990). Vanadium accumulates in considerable amounts in particular blood cells of ascidians (Scippa et al., 1982, 1988; Michibata et al., 1987) and has been investigated in several studies, being a powerful inhibitor of many enzymatic activities (Cantley et al., 1977; Stankiewicz et al., 1995). Vanadium modifies DNA synthesis and repair (Sabbioni et al., 1983; Smith, 1983) and is able to form DNA–protein crosslinks and produce DNA strand breaks in human leukocytes in vitro (Birnboim, 1988; Rojas et al., 1996). Reports on its mutagenicity (Kanamatsu et al., 1980), genotoxicity (Owusu-Yaw et al., 1990; Altamizam-Lozano et al., 1999), insulin mimicry (Orvig et al., 1995) and antitumor activities (Djordjevich, 1995) have been published. Moreover, vanadium at relatively high concentrations has been shown to be cytotoxic, but only mildly, if at all, mutagenic (Leonard and Gerber, 1994).

Our report analyses the potential mutagenic effects of a brief exposure to sodium metavanadate, at concentrations not altering cell viability and proliferation, on Mel cell erythropoiesis. The role of the efficiency of DNA repair on permanent fixation of damage during the brief pulse of metavanadate was evaluated using two compounds well known for their opposite effects on DNA repair, hydroxyurea (HU) and vanillin, which inhibit and stimulate DNA repair, respectively.

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Materials and methods

Cell line and culture conditions
DS19, a clone of mouse Friend erythroleukemia (Mel) cells, was used. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL) supplemented with 550 U/ml penicillin, 75 U/ml streptomycin (Sigma) and 7.5% fetal calf serum (FCS) (Flow Laboratories). The cultures were incubated in a humidified atmosphere at 37°C in 10% CO₂. Mel cells were seeded at a density of 1x10⁶ cells/ml in a 5 cm Petri dish; proliferation and viability were followed daily by counting viable cells that excluded trypan blue dye (Gurr) in a Burker chamber.

Determination of erythroid differentiation
Erythroid differentiation was induced by addition of 2% dimethylsulfoxide (DMSO) to the complete culture medium (induction medium) and was monitored by following the accumulation of Hb in each cell with the benzidine cytochemical test (Foresti et al., 1990) and in the cell lysates by spectrophotometric evaluation of Hb, assuming that ε₄₁₅ nm per mM oxyhemoglobin is 125.

Vanadium treatments during DMSO-induced differentiation of Mel cells
Sodium metavanadate (NaVO₃, mol. wt 121.9) (Sigma) was used. A stock solution of 5 mg/ml metavanadate was prepared by dissolving 5 mg of the salt in 1 ml of 1.5 M HCl; working solutions of metavanadate were then prepared by diluting the stock solution with double distilled sterile water.

Two different periods of exposure to metavanadate were tested. In one metavanadate was always present in the culture medium of Mel cells; this is referred to as chronic exposure. In the other Mel cells were pelleted after a 5 h exposure to vanadium and washed several times with DMEM containing FCS and DMSO. Finally the cells were allowed to grow in the induction culture medium in the absence of vanadium salt for 5 days. The metavanadate pulse lasted 5 h, the whole first period of Mel cell sensitivity to mutagens (Foresti et al., 1997).

Inhibition and stimulation of DNA repair synthesis
DNA synthesis was inhibited by adding 1 mM HU to the culture medium of Mel cells for 2 h, starting 3 h from the start of induction, in the presence or not of 0.1 µg/ml metavanadate. HU was then washed from the cells using warm complete induction medium and the Mel cells were allowed to progress to erythropoiesis for 5 days. DNA repair was stimulated by adding 5 µg/ml vanillin to the Mel cell induction culture medium during the 5 h period of mutagen sensitivity, in the presence or not of 0.1 µg/ml metavanadate, and then the cells were treated as above. In some experiments Mel cells were firstly exposed to 0.1 µg/ml metavanadate for 5 h, as reported, then metavanadate was washed out and the cells were resuspended in warm induction medium plus vanillin.

[³H]Deoxythymidine ([³H]TdR) incorporation
Mel cells, plated at a density of 1x10⁶ cells/ml in induction medium, were incubated with 1 µCi/ml [³H]thymidine (53 mCi/mMol) during the 5 h treatment with 0.1 µg/ml metavanadate plus 1 mM HU or 5 µg/ml vanillin. Thymidine incorporation was measured in the TCA-insoluble precipitate, as reported (Foresti et al., 1993).

All experiments were performed in duplicate or triplicate and were repeated at least three times.

Statistical test
Student’s t-test for paired values was applied for statistical analysis of the data.

Results
Figure 1A reports the growth curve for Mel cells exposed to metavanadate during the first 5 h from the start of induction and then grown in culture medium without metavanadate. Metavanadate concentrations of 0.1 and 0.01 µg/ml did not alter the growth kinetics of the treated cells or of their progeny, while at 10 µg/ml a 50% reduction in growth was observed. The inset reports the number of Mel cells chronically exposed to metavanadate for 3 days; as expected, a severe inhibition of proliferation was observed.

Figure 1B depicts the effect of a 5 h pulse of metavanadate at doses ranging from 0.0001 to 10 µg/ml on Mel cell erythroid differentiation. A metavanadate concentration of 1 or 10 µg/ml inhibited differentiation. Among the sublethal doses only 0.1 µg/ml metavanadate affected erythroid differentiation, which was inhibited by 23%. At concentrations of 0.01, 0.001 and 0.0001 µg/ml no apparent alterations were observed. A chronic exposure to 0.1 µg/ml metavanadate reduced Mel cell
Metavanadate and Mel cell differentiation

Fig. 2. Effects of metavanadate on Hb accumulation. Mel cells were seeded at a density of 1×10^5 cells/ml in DMEM plus 7.5% FCS, 2% DMSO and the indicated concentrations of metavanadate. After 5 h incubation metavanadate was washed out, as reported in Materials and methods, then the cells were incubated in induction medium without metavanadate. Hb was determined in the total cell lysates at day 5 of induction.

Table I

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<th>%C</th>
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(a) Mel cells were seeded at the density of 1×10^5 cells/ml in DMEM plus 7.5% FCS and metavanadate at the indicated concentrations. After 5 h incubation metavanadate was washed out and the cells were induced to differentiate in DMEM, 7.5% FCS and 2% DMSO. Induced cells were determined at day 5 of induction.

Afterwards we investigated whether the damage induced by low-level exposure to metavanadate during the first 5 h of induction of erythroid differentiation was stably inherited by the cell progeny. For this purpose, after treatment the Mel cells were washed to eliminate the vanadium salt and DMSO and then cultured and routinely propagated for 4 months in DMEM plus 7.5% FCS. Aliquots of cells were then induced to undergo erythroid differentiation with 2% DMSO. The tested cells showed a reduced capacity to differentiate, which depended on the metavanadate dose that was initially used to mutagenize the parental Mel cells 4 months before. In fact, the number of differentiated cells was 64% of the controls in cells derived from those exposed to 0.1 µg/ml metavanadate, while it was 88 and 100% when the lower concentrations of 0.001 and 0.0001 µg/ml metavanadate was used (Table Ib).

The role of DNA repair synthesis during the 5 h treatment of Mel cells with metavanadate was then studied using two molecules well known for their effects on DNA repair. HU is an inhibitor of ribonucleotide diphosphate reductase, which

Fig. 3. Modulation by HU and vanillin of the effects of low-level exposure to metavanadate on Mel cell differentiation. Mel cells were seeded at the density of 1×10^5 cells/ml in DMEM plus 7.5% FCS, 2% DMSO and 0.1 µg/ml metavanadate in the presence or not of 1 mM HU or 5 µg/ml vanillin. After 5 h incubation metavanadate, HU and vanillin were washed out of the culture medium, as reported in Materials and methods, then Mel cells were permitted to progress into erythropoiesis in the DMEM, 7.5% FCS, 2% DMSO induction medium. In one experiment 5 µg/ml vanillin was added to the culture medium of Mel cells after the metavanadate treatment. The number of induced cells positive in the benzidine test (%B⁺) was determined at day 5 of induction. Standard deviations are reported.
blocks DNA repair synthesis by depletion of the deoxynucleotide pools (Collins and Oates, 1987). Vanillin is an antigenotoxic, desmutagenic agent that stimulates DNA repair synthesis, mainly through its antioxidant activity, and/or recombinational repair. The antigenotoxic effect of vanillin seems to be related to the type of induced DNA lesion and to the type of repair process involved in its correction (Kuroda and Inhoue, 1988; Santos et al., 1999).

Therefore, Mel cells were first exposed to 0.1 μg/ml metavanadate plus 1 mM HU or 5 μg/ml vanillin during the first 5 h of induction, in order to modulate DNA repair during the metavanadate treatment. They were then cultured in induction medium not including metavanadate and DNA repair modulators. The cells were examined for the level of differentiation reached at day 4 of culture. Figure 3 shows that, as reported above, metavanadate alone inhibited erythropoiesis, but the effect on differentiation was different depending on whether HU or vanillin was present during the 5 h mutagen pulse. The progeny of cells exposed to metavanadate plus HU showed a lower capacity to differentiate, as compared with those treated only with metavanadate. In contrast, cells derived from those exposed to metavanadate plus vanillin differentiated to the same extent as the controls. Similar results were obtained when vanillin was added to the culture medium of Mel cells after the 5 h exposure to metavanadate, in order to exclude interaction between the two molecules. HU and vanillin did not alter Mel cell differentiation and proliferation (data not shown).

DNA repair synthesis during the 5 h exposure of Mel cells to 0.1 (Figure 4A) or 0.01 μg/ml (Figure 4B) metavanadate plus 1 mM HU or 5 μg/ml vanillin was also directly followed by measuring [3H]TdR incorporation into Mel cells, and the results are reported in Figure 4. Metavanadate alone at 0.1 μg/ml stimulated [3H]TdR incorporation by 130% with respect to the controls, while during a pulse with vanillin incorporation increased to 200%. HU, alone and with metavanadate, caused 49 and 62% inhibition, respectively, of thymidine incorporation with respect to untreated control Mel cells.

Fig. 4. Modulation by HU and vanillin of the effects of low-level exposure to metavanadate on Mel cell thymidine incorporation. (A) Mel cells were seeded at a density of 1×10^6 cells/ml in DMEM plus 7.5% FCS, 2% DMSO, 1 μCi/ml [3H]TdR and 0.1 μg/ml metavanadate plus 1 mM HU or 5 μg/ml vanillin. After 5 h incubation the cells were processed for thymidine incorporation. Standard deviations and P values determined with the Student t-test for paired values are reported. (B) Mel cells were treated as in (a) except that 0.01 μg/ml metavanadate was used. ***, 0.01 < P > 0.0001, highly significant; **, 0.01 > P > 0.05, low significance; *, P > 0.05 no significance.

Discussion

Erythropoiesis can be induced in vitro in Mel cells by DMSO addition to the culture medium. DMSO immediately triggers a series of events leading to the reorganization of the chromatin necessary to sustain erythroid differentiation (Terada et al., 1977). Mel cells are parasyynchronized by DMSO into the G1/S phase of the cell cycle during the first 6–10 h after the start of induction (Terada et al., 1977). This period represents the earlier of the two time windows in the cell cycle of differentiating Mel cells during which the cells can be easily mutagenized (Foresti et al., 1997).

It is well known that packaging DNA into chromatin affects the accessibility to DNA of proteins involved in transcription, repair and replication (Smerdon, 1989; Smerdon and Conconi, 1999). Therefore, it was previously suggested that during erythroid-mediated chromatin remodeling the DNA repair machinery, working at full efficiency in zones where chromatin has already been reshaped, can be hampered by local remodeling (Foresti et al., 1997). As a consequence, damage will immediately be fixed in Mel cell DNA during the following S phase, because the DNA repair machinery has not recognized the induced damage as an alteration (Foresti et al., 1993).

It appeared interesting to understand this cell cycle-related sensitivity, which allows detection of the activity of mutagens at doses not altering cell viability and proliferation. For this purpose, the role of the efficiency of DNA repair in Mel cells was evaluated during the initial period of erythropoiesis induction, using sodium metavanadate as a model mutagen. As previously mentioned, many reports on vanadium mutagenicity have been published, which, however, utilized high doses and very long exposures. Though such conditions could affect occupational workers, they might not be the most commonly found in the environment. The diffusion of vanadium in the environment as an industrial contaminant is increasing and its use as an insulin mimic has been proposed (Orvig et al., 1995). Therefore, the genotoxic effect of vanadium on Mel cells was detected under conditions that can be more commonly found, such as brief exposures at low doses. English et al. (1983) showed that a 72 h exposure to 20 μM ammonium vanadate blocked erythroid induction of Mel cells. In our work...
these data were confirmed, using 10 µg/ml metavanadate and, in addition, a 25 times lower dose with a 5 h exposure.

The aim of the present report was to analyse the ability of Mel cells to progress to erythropoiesis. This is a complex multigenic cellular function and, hence, the presence of several targets was considered useful to phenotypically increase the effects of a low-dose exposure to the mutagen.

The initial brief low-level exposure to 0.1 µg/ml metavanadate during induction permanently damaged the capacity of treated cells and their progeny to differentiate. The same 5 h treatment was also tested in a different period of the cell cycle. In this case Mel cells were first exposed to 0.0001, 0.001 or 0.1 µg/ml metavanadate and then treated with DMSO to induce erythropoiesis. No inhibition of differentiation was observed because of cell asynchrony during treatment, in agreement with previous results obtained with other mutagens (Foresti et al., 1990).

The occurrence of cell clones permanently altered in the erythroid pathway was demonstrated in the progeny of Mel cells initially treated with metavanadate for only 5 h from the start of induction and then routinely propagated for 4 months in culture medium in the absence of metavanadate and inducer. Periodically aliquots of cells were harvested and switched to erythroid differentiation with DMSO. The persistence of the same reduced capacity of these cells to undergo erythropoiesis indicated that endogenous DNA lesions induced by low-level exposure of the parental cells to metavanadate some months before were never properly repaired, but were instead stably inherited as new cell properties.

When vanadium in the +4 or +5 oxidation state is given to animals vanadium is found exclusively in the vanadyl +4 form (Sakurai et al., 1980). Sakurai (1994) showed that the mechanism of vanadium-dependent toxicity and antineoplastic action is due to DNA cleavage by hydroxyl radicals generated in living systems. Shi et al. (1996) reported the role of molecular oxygen in the mechanism of vanadium(IV)-induced 2'-deoxyguanosine hydroxylation to form 8-hydroxy-2'-deoxyguanosine and showed that vanadium also caused molecular oxygen-dependent DNA strand breaks.

In order to evaluate the efficiency of the DNA repair machinery during low-level exposure to 0.1 µg/ml metavanadate two molecules, HU and vanillin, were used, which reportedly block and stimulate DNA repair, respectively. Mel cells pulsed for 5 h with metavanadate plus HU or metavanadate plus vanillin during erythropoiesis induction showed a higher percentage of modified cells or maintenance of the wild-type phenotype, respectively. The antimitogenic effect of vanillin was observed not only during the metavanadate plus vanillin treatment but also when vanillin was added to the culture medium of Mel cells after 5 h exposure of the cells to metavanadate, so that no interaction between the two molecules occurred. Increased uptake of thymidine during exposure to the salt showed that metavanadate stimulated DNA repair synthesis, however, this repair did not appear to be effective in removing all DNA lesions induced by the metal, as cell clones permanently altered in differentiation capacity were obtained. When DNA repair was blocked by HU a correlation between greater metavanadate-induced inhibition of Mel cell differentiation and decreased efficiency of DNA repair was observed. Conversely, when DNA repair was stimulated by vanillin, either during mixed exposure with vanadium or after mutagen treatment, the decrease in the inhibitory effects of metavanadate on differentiation appeared to be related to the higher efficiency of DNA repair caused by vanillin.

If a compound is able to damage DNA it will reasonably damage the target DNA sequences accessible to its action. Our observations on the effects of metavanadate indicate that the activity of a mutagen can depend not only upon its concentration, but also on the efficiency of the DNA repair systems activated to correct the induced damage, as well as on the extent to which damage can actually alter the function of the modified DNA sequence. The absence of effects on differentiation of 0.001 and 0.0001 µg/ml metavanadate does not necessarily exclude the possibility that metavanadate did not damage the erythroid pathway. Two possible events may have occurred: the alteration is repaired and/or the induced lesions accumulated as silent mutations, incapable of altering erythropoiesis, probably at the third base of a triplet or in intronic regions or repeated DNA sequences.

In conclusion, our results show that: (i) a brief, cell cycle-dependent, low-level exposure to metavanadate can selectively mutagenize the functions involved in the Mel cell erythroid pathway, leaving apparently unaltered all the functions controlling cell viability and proliferation; (ii) the efficiency of cell activities involved in the removal of DNA damage during mutagenesis plays a fundamental role in the fixation of DNA damage.

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References


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