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Short communication

O^6-Alkylguanine-DNA alkyltransferase content in synchronised human cancer cells*

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Summary. The DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (AT) was analysed in the human ovarian-cancer SW626 cell line and in the human promonocytic leukemia U937 cell line following their synchronisation with low non-toxic concentrations of methotrexate. In SW626, AT increased in the early S phase of the cell cycle and then declined during progression of the S phase to levels found in the G1 phase of unsynchronised cells. In contrast, at the G1/S-phase boundary and in the S phase, U937 cells showed a lower AT content than did exponentially growing unsynchronised cells. In addition, AT activity was greatly reduced in resting U937 cells but was not reduced appreciably in resting SW626 cells. The results of these studies indicate that AT fluctuations do not follow a constant pattern during the cell cycle of different cell lines.

Introduction

The alkylation of guanine-O^6 in DNA is one of the most carcinogenic lesions produced by alkylating agents [12] and is also important for the cytotoxicity and antitumor activity of some anticancer agents, such as chloroethylnitrosoureas [9] and methyltriazenes [2]. The enzyme O^6-alkylguanine-DNA alkyltransferase (AT) is a DNA repair protein that acts as an alkyl acceptor, transferring the alkyl group from the oxygen atom of the guanine to a cysteine residue of the protein [4, 5]. The role of AT in modulating the cytotoxic potential of alkylating agents is supported by a considerable amount of experimental evidence [5, 15].

Cancer cell lines displaying a low AT content are more sensitive to nitrosoureas and methyltriazenes than are those containing high levels of the enzyme [2, 5]. Some physical and chemical factors that can influence cellular levels of AT have been identified [7, 11, 14, 17]. However, only scant information is available on the fluctuation of cellular AT levels during the cell cycle. Using a synchronisation method [16] previously set up in this laboratory, we investigated this aspect in two human cancer cell lines.

Materials and methods

Materials. Methotrexate (MTX) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). Propidium iodide (PI) and RNAse were purchased from Calbiochem Corporation. RPMI-1640 cell-culture medium and fetal bovine serum (FBS) were obtained from Gibco (UK) and Flow Laboratories (Scotland), respectively. Calf-thymus DNA was obtained from Sigma (St. Louis, Mo.). *N*-[^{3}H]-methyl-*N*-nitrosourea (MNU; sp. act., 13.9 Ci/mmol) was purchased from Amersham (UK).

Cells and culture conditions. Human ovarian-carcinoma SW626 cells were grown as a monolayer, whereas human histiocytic lymphoma U937 cells were grown in suspension in RPMI-1640 medium supplemented with 10% FBS under standard culture conditions described in detail elsewhere [16].

Cell synchronisation. Synchronisation protocols for the two cell lines have been described elsewhere [16]. In short, exponentially growing SW626 cells were incubated with 0.08 μM MTX for 24 h, whereas the U937 cell line required exposure to 0.04 μM MTX for 16 h under standard culture conditions. After incubation, the synchroniser-containing medium was removed, the cells were washed with phosphate-buffered saline (PBS) and fresh medium supplemented with 10% FBS was added. At different intervals, synchrony was checked by flow-cytometric methods.

Flow-cytometric analyses. Uniparametric conventional cell-cycle analyses using PI [8] were performed at various times using an FACS Star Plus instrument (Becton Dickinson) coupled to a Consort 30 computer.

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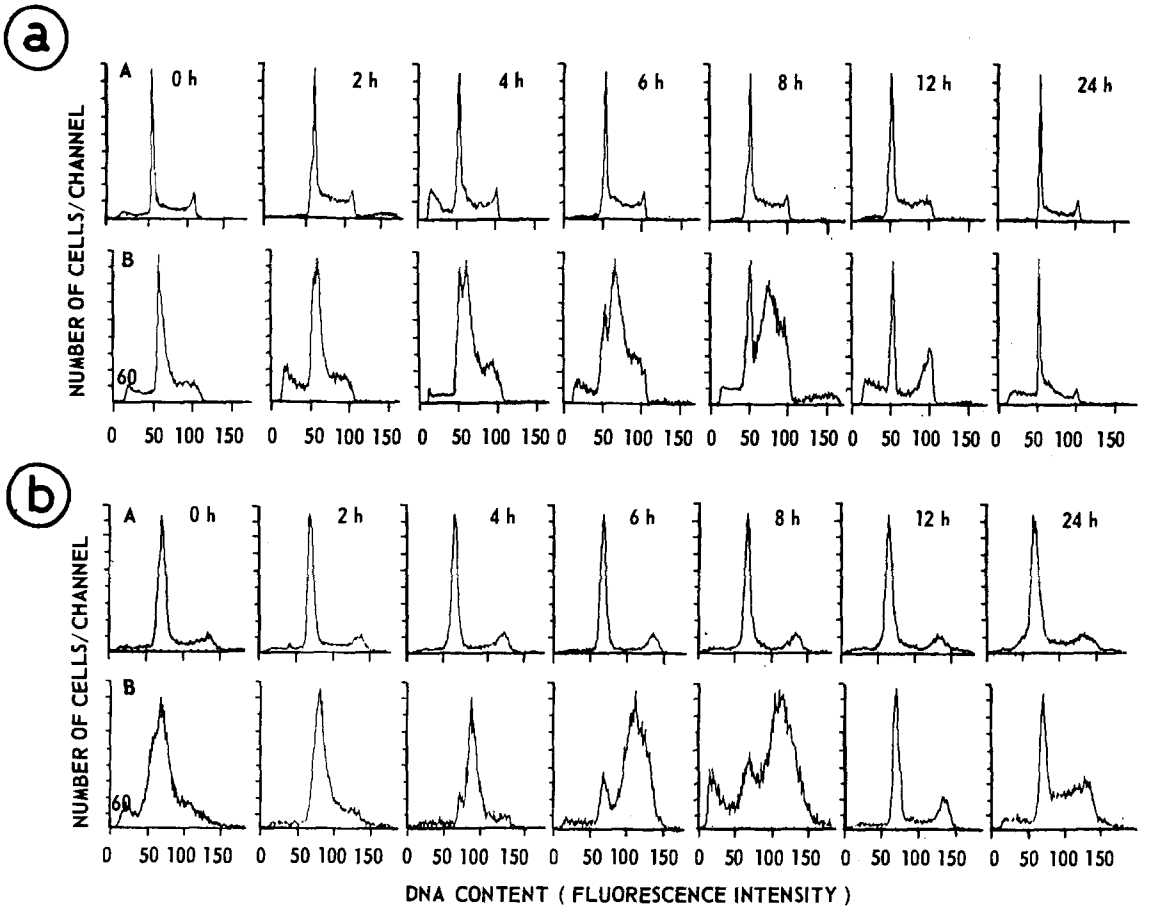

Fig. 1 a, b. Cell-cycle-phase distribution of **a** U937 and **b** SW626 cells obtained by uniparametric flow-cytometric analysis of DNA content (propidium iodide red-fluorescence intensity in fluorescence units) at various times after MTX washout. On the ordinate are shown the number of cells corresponding to the fluorescence units. *A*, Exponentially growing, untreated control cells; *B*, cells synchronised with MTX

Detailed flow-cytometric data on the synchronised populations has previously been reported in detail [16].

Preparation of cellular extract. The SW626 cells were harvested by a cell scraper and resuspended in PBS, whereas the U937 cells were centrifuged and resuspended in PBS. Crude cellular extract was obtained by sonication of the pellets in 10 mM TRIS-HCl buffer (pH 7.4) containing 1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM methylphenylsulphonyl fluoride [3]. Debris was removed by centrifugation (700 *g* for 15 min), and the supernatant was used for determination of AT activity. Total protein contents were measured according to the method of Lowry et al. [13]. Estimation of total cellular protein at different intervals did not show significant differences between exponentially growing, untreated control cells and cells that had been synchronised with low levels of MTX [mean protein content ± SD expressed in milligrams per 50 million cells estimated: U937 control, 3.97 ± 0.12; synchronised, 3.93 ± 0.23 (recovery at time zero, 4.10; 2-h recovery, 3.85; 4-h recovery, 3.94; 6-h recovery, 4.05; 24-h recovery, 4.28); SW626 control; 2.56 ± 0.83; synchronised, 2.40 ± 0.47 (recovery at time zero, 2.03; 2-h recovery, 2.30; 4-h recovery, 2.05; 6-h recovery, 2.43; 24-h recovery, 2.13)]. The results are consistent with our previous finding [16] that synchronisation of cells with low doses of MTX does not affect intracellular total protein or nucleotide pools.

Preparation of substrate. [^{3}H]-Methylated substrate was prepared according to the method of Wiestler et al. [19] using 4 mg calf-thymus DNA, which was incubated with 0.6 mCi [^{3}H]-MNU for 60 min at 37° C in 10 mM TRIS-HCl buffer (pH 8.3).

Determination of AT activity. AT activity was determined as previously described [1]. Briefly, crude cellular extract was incubated with substrate DNA at 37° C for 60 min. After precipitation with NaCl and ethanol at 4° C, the DNA was hydrolysed with 0.1 *N* HCl at 70° C for 45 min. O^6-Methylguanine removed from the hydrolysate was monitored by high-performance liquid chromatography (HPLC). Hydrolysate was injected onto a 25-cm Partisil 10 SCX cation-exchange column (Whatman). Elution was carried out at a flow rate of 1 ml/min using a buffer whose composition was changed from 0.02 M ammonium formate (pH 4) in 6% methanol to 0.2 M ammonium formate (pH 4) in 8% methanol over 30 min using a gradient programme of the Beckman HPLC system. The HPLC profile of the acid hydrolysate showed peaks of guanine, 7-methylguanine (7-meG), adenine and O^6-methylguanine. 7-meG was the adduct found in the highest concentration, but its content was the same at all intervals tested. However, the amount of O^6-methylguanine removed was different at various sampling times; this reflected variations in O^6-methylguanine repair by the crude cellular extract used in the present study. O^6-methylguanine was eluted at 21 min after injection and was collected in scintillation vials. Radioactivity was counted by liquid scintillation spectrometry. AT activity was expressed in femtomoles of O^6-methylguanine removed per milligram of cellular protein as previously described [1].

Results

Figure 1 shows the cell-cycle distribution of U937 and SW626 cells at several intervals after pretreatment with low concentrations of MTX (0.04 and 0.08 μM, respectively). As previously reported [16], at these concentrations, MTX pretreatment of these two cell lines caused a reversible accumulation of cells at the G1/early S-phase

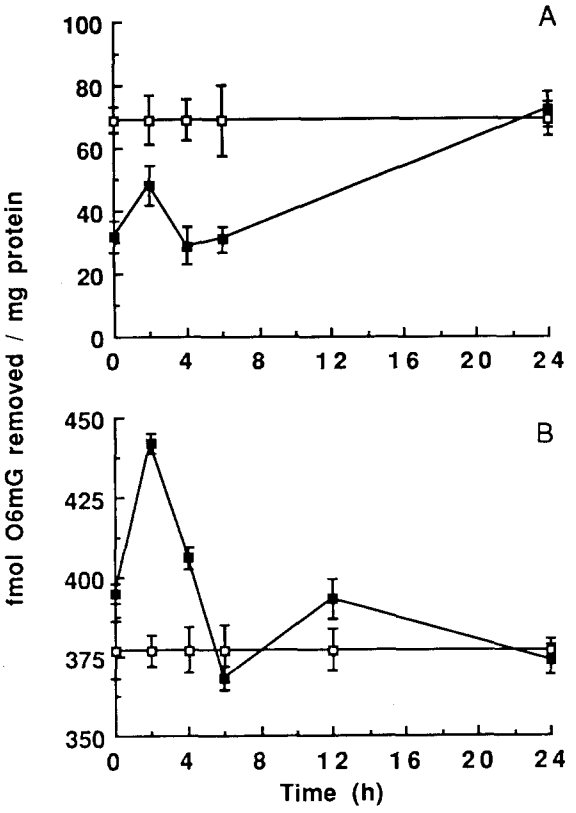

Fig. 2 A, B. O^6-Alkylguanine-DNA alkyltransferase (AT) levels in **A** U937 and **B** SW626 cells. On the ordinate are shown the estimated AT activities. On the abscissa are given the times of sampling after synchronisation with MTX for specific periods. *Open symbols*, exponentially growing, untreated control cells; *closed symbols*, cells synchronised with MTX. Points represent mean values for at least 3 independent estimations; bars indicate the SEM

boundary. On removal of the MTX, the cells cycled as a synchronised population through various phases of S, G2 and mitosis. MTX treatment does not affect cell growth, clonogenicity or the duration of the S phase and can be considered a reliable method of synchronisation.

Figure 2a shows that the levels of AT decreased in U937 cells when a high proportion of cells were in the S phase (between 0 and 6 h of MTX washout). Interestingly, in U937 cells, AT levels showed a sudden but consistent increase (by approximately 54%) at 2 h after MTX washout, when a majority of the cells were in the early S phase; the AT content then declined and remained significantly lower than that observed in exponentially growing control cells. At 24 h after MTX washout, when cells were no longer synchronised, AT levels became comparable with those found in control cells growing logarithmically. When U937 cells had become quiescent by growing to their maximal density, AT was undetectable (data not shown).

AT levels in SW626 cells followed a different pattern (Fig. 2b), increasing by approximately 11% at 2 h after release from MTX, at which time the cells were enriched in the early S phase. The AT content then declined gradually and became comparable with the control value at between 6 and 12 h, when a large proportion of the cells were in the late S and G2M phases of the cell cycle. AT levels remained comparable with those found in exponentially growing cells for 24 h after MTX washout (when the results of cell-cycle analysis corresponded to those for a control population). Since SW626 cells cannot be made quiescent through growth to a high density, we could not evaluate their AT content under these conditions. These cells lack contact inhibition, and serum deprivation does not stop their proliferation completely [16]. However, when SW626 cells were kept under confluent conditions in the absence of serum for 1 week, the rate of DNA synthesis was much lower (approximately 85% inhibition) than that observed in logarithmically growing cells, whereas the AT levels did not vary significantly from those found in exponentially growing cells (data not shown).

Discussion

In this study we found a discrepant pattern for AT levels during the cell cycles of two human cancer cell lines that had been synchronised with low concentrations of MTX [16]. In particular, human hystiocytic lymphoma U937 cells, which displayed relatively low levels of AT, showed a further decrease in the enzyme levels in S-phase cells. In contrast, in SW626 human ovarian-cancer cells, which exhibited considerably higher AT levels, a significant increase was observed in the enzyme levels in S-phase cells, particularly at the beginning of the S phase.

To our knowledge, only two reports have been published on the influence of the cell cycle on AT levels: one involved human skin fibroblasts [10] and the other, mouse embryo cells [6]. Kim et al. [10] reported that normal human skin fibroblasts made quiescent by serum deprivation displayed constant levels of AT. After stimulation of these cells with serum, a significant increase in the enzyme level was observed; this increase was noted before the induction of DNA replication had become evident. AT levels reached a maximum at 21 h after serum stimulation and then declined to baseline values. In contrast, in hypermutable Bloom's-syndrome fibroblasts, serum stimulation induced DNA replication but caused no change in AT levels, suggesting that AT expression is not normally regulated in Bloom's-syndrome fibroblasts and that this might contribute to the increased frequency of spontaneous mutation of these cells [10]. Since the increase observed in AT levels in normal human fibroblasts following serum stimulation remained evident for many hours, the authors suggested that there was a difference in the AT content of cycling vs non-cycling cells. No analysis of variations among the different phases of the cell cycle was attempted. The present findings indicate that resting cells display lower AT levels. U937 cells, which could be made quiescent in the Go/G1 phase through growth to their maximal density, showed a clear-cut decrease in levels of AT.

Dunn et al. [6] reported on their investigation of AT levels in partially synchronised C3H/10T1/2 mouse embryo cells. These authors found that AT levels had reduced prior to the beginning of the S phase and remained lowered throughout the S phase, becoming normal during the G2 phase. The data we obtained in U937 cells are essentially in line with this observation. These data have important im-

plications for the methylating and chloroethylating agents, which could show higher efficacy in cells in the S phase because of their less efficient repair of O^6-guanine alkylation. Work is in progress to verify this point. On the other hand, we observed a different pattern in SW626 cells. The discrepant results obtained in the two cell lines suggest that the regulation of AT expression during the cell cycle is not the same in all cells. Since we analysed two cancer cell lines, one of which was derived from a human lymphoma (U937) and the other, from a human ovarian epithelial cancer (SW626), it may be that the regulation of AT during the cell cycle varies in different cell types. Additional studies on other hematological and epithelial normal and cancer cell lines are needed before definitive conclusions can be drawn on this point. The availability of the recently cloned human gene encoding human AT [18] will make it possible to investigate the mechanisms of regulation of this gene during the various phases of the cell cycle in different cell lines at the molecular level.

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