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Environmental Toxic Agents on Genetic Material and Cellular Activity IV. Novobiocin-Mediated Inhibition of DNA Repair Synthesis in Synchronized Chinese Hamster Ovary Cells

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The effect of novobiocin (NOV), an inhibitor of topoisomerase II, on ethyl methanesulfonate (EMS)- or bleomycin (BLM)-induced DNA repair synthesis was examined during the cell cycle of Chinese hamster ovary (CHO)$-K_1$ cells. Three assays were employed in this study: cell survival, alkaline elution and unscheduled DNA synthesis. EMS was effective at killing CHO cells in G_1 phase, whereas BLM preferentially killed cells in G_2 and S phases. EMS induced the much more amount of DNA damage in G_1 phase, while BLM induced in G_2 phase than the other phases. The both of pre- and post-treatment with NOV inhibited EMS- or BLM-induced DNA repair synthesis in G_1 and G_2 phases, and pretreatment with NOV inhibited more effectively than the post-treated group. These results suggested that CHO cells exhibited a differential sensitivity to cell lethality and DNA damage in relation to cell cycle according to used chemical agents, and that DNA topoisomerase II participated in an initial stage of DNA repair.

INTRODUCTION

DNA topoisomerase II was discovered relatively recently (Liu *et al.*, 1980) and have been known to function in DNA replication (Jazwinski and Edelman, 1984) and repair (Mattern *et al.*, 1982). It alters the topology of nucleic acids passing one double-stranded segment of DNA through a transient break made

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in a second helical segment (Wang, 1985). The results concerning the functions at this enzyme can be obtained using this enzyme inhibitor. Novobiocin (NOV) inhibits a type II eukaryotic DNA topoisomerase (Liu *et al.*, 1980) and also inhibits an early step of excision repair in mammalian cells (Collins and Johnson, 1979; Mattern and Scudiero, 1981).

In recent studies, it was suggested that topoisomerase was present not only in the mitotic phase of cell cycle, but also in G_1 and S phases of the cell cycle (Markovits *et al.*, 1987). And there was a report that topoisomerase II activity increased in re-generating rat liver (Duguet *et al.*, 1983), it suggested a possible correlation between the activity of mammalian topoisomerase II and cell proliferation. The study of Tricoli *et al.* (1985) showed no differences in topoisomerase II activity in proliferating versus non-proliferating mouse embryo fibroblast cells, nor in the G_1, S and M phases of synchronized cells. Sullivan *et al.* (1986) found that topoisomerase II DNA cleavage activity was several-fold higher in exponential phase versus plateau phase CHO cells but not strinkingly in HeLa or L1210 cells.

The present studies, therefore, were to elucidate the role of topoisomerase on the repair process of DNA damage induced by ethyl methanesulfonate (EMS) or bleomycin (BLM) in relation to different phases of the cell cycle, and to investigate the cytotoxicity of these drugs correlated with the production of DNA lesions in different phases of the cell cycle.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary (CHO)-K_1 cells were used throughout this investigation. Monolayer cultures of this cell line were grown at 37°C in humidified 5% CO_2 incubator using Eagle's minimum essential medium (MEM; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% newborn calf serum and gentamycin (50 μg/ml).

Chemical Treatment

Ethyl methanesulfonate (EMS, Tokyo Kasei Co., Tokyo, Japan) or bleomycin (BLM, Nihon Kayaku, Japan) was dissolved in the serum-free medium prior to use and exposed to cells at 37°C for desired time. Novobiocin (NOV, Sigma Chemical Co., St. Louis, MO), an inhibitor of DNA topoisomerase II, was dissolved in distilled water and further diluted to working concentrations. The cells were treated to this inhibitor for 1 hour.

Cell Synchronization

Monolayer cultures of CHO cells were synchronized with a slight modification of the method developed by Terasima and Tolmach (1963) using mitotic selection. the degree of synchronization achieved was determined by the rate of DNA synthesis by autoradiography. For this purpose, the cells were pulse labeled with ^{3}H-thymidine (Specific activity; 77.9 Ci/mM, Amersham Co., England) for 10 minutes at a final concentrationof 1 μCi/ml. Labeling with ^{3}H-thymidine was terminated by washing the cells three times with PBS containing 100 μg/ml unlabeled thymidine. Autoradiograms were prepared and the degree of synchronization was measured by the labeling index of synchronized cell population.

Survival Studies

Survival test was performed according to Terasima *et al.* (1972) with minor modifications. For colony formation assays, CHO cells were in logarithmic growth phase. The synchronized cells were exposed to drug for desired time and then washed three times with PBS. Single-cell suspensions were prepared with 0.05% trypsin-EDTA. For controls and drug treatment, 300 cells were plated per 60 mm diameter tissue culture dishes (Nunclon, Denmark). The plated cells were incubated for 7-8 days in growth medium and then fixed 2 times with Carnoy's solution. Colonies were stained with 4% Giemsa and counted.

Alakline Elution Experiments

Alkaline elution was performed essentially according to Kohn *et al.* (1976) with minor modification. Cells were labeled with 0.2 μCi/ml of ^{3}H-thymidine for 24 hours and then exposed to chemicals. The cells harvested with cold PBS-Merchant solution (150 mM NaCl, 4.28 mM K_2HPO_4, 0.71 mM KH_2PO_4), and filtered onto 2 μm pore size polycarbonate filter (Nuclepore Co., Pleasanton, CA), and lysed with lysing solution (2% SDS, 0.1 M Glycine, 0.025 M Na_2-EDTA, pH 10.0). Cells were eluted in the dark with eluting solution (30 mM tetrapropylammonium hydroxide, 0.02 M EDTA, 1% SDS, pH 12.1) at a flow rate of 0.035 ml/min. Fractions were collected at 90 minutes interval. The radioactivity remaining on filter was plotted against elution time.

Unscheduled DNA Synthesis

The experiments involving unscheduled DNA synthesis were carried out as described by Cleaver and Thomas (1981) with minor modification. CHO cells grown on coverslips in plastic petridishes were exposed to chemicals. The cells were then labeled with 10 μCi/ml ^{3}H-thymidine for 1 hour after treatment with inhibitor. Labeling with ^{3}H-thymidine was terminated by washing the cells three times in cold Hank's balanced salt solution (HBSS) containing 100 μg/ml of unlabeled thymidine. Autoradiograms were prepared by using Kodak NTB liquid nuclear track emulsion. Silver grains over nuclei of evenly and lightly labeled cells were counted.

RESULTS

CHO cells synchronized by using mitoitc selection method were used throughout this investigation. The degree of synchronization was established in terms of the labeling index in DNA synthesis. The generation time of CHO cells is 16 hours: the durations of G_1, S, G_2 and M-phases of CHO cells occupy about 4, 9, 2, and 1 hour, respectively (data not shown).

The dose response of EMS or BLM on survivals in relation to cell cycle in CHO cells is shown in Fig. 1. The stage sensitivity of cell survival is shown in G_1 phase in EMS-treated group (1-10 mM), while that is shown in G_2 and S phases in 10 μg/ml BLM-treated group.

Figs. 2 and 3 show the effect of 1 mM NOV on DNA singlestrand breaks induced by 120 mM EMS or 800 μg/ml BLM in synchronized CHO cells. The pre-treatment with NOV inhibited the rejoining of DNA single-strand breaks by EMS or BLM in G_1 and G_2 phases, and resulted in the much more accumulation of DNA single-strand breaks than that of the EMS or BLM alone treated group. And post-treatment with NOV also inhibited the rejoining of DNA single-strand breaks by EMS or BLM in G_1 and G_2 phases.

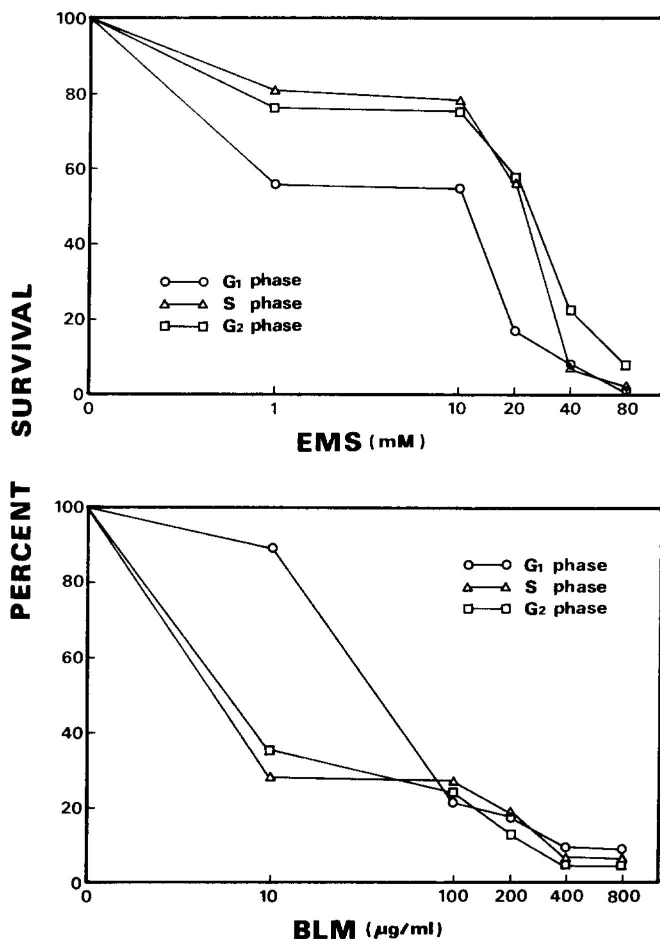

Fig. 1. **Dose response curve of CHO cells. Survivals were assayed by exposure of cells to EMS or BLM for 1 hour in various phases.**

Fig. 4 represents the effect of unscheduled DNA synthesis induced by EMS or BLM in synchronized CHO cells. In G_1 and G_2 phases, the amounts of unscheduled DNA synthesis induced by EMS or BLM are increased with dose increment and reached almost plateau at 5 mM EMS or 40 μg/ml BLM. However, these results indicate that the stage sensitivity of unscheduled DNA synthesis is shown in G_1 phase of EMS-treated group and G_2 phase of BLM-treated group.

Figs. 5 and 6 show the effect of NOV on unscheduled DNA synthesis induced by EMS or BLM in synchronized CHO cells. As shown in the figures, the amount of unscheduled DNA synthesis was increased in the group pre-or post-treated with NOV as compared with EMS or BLM alone treated group

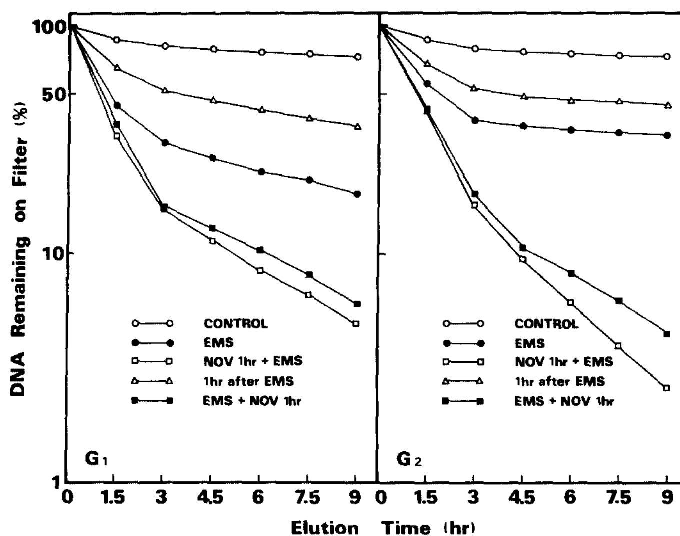

Fig. 2. Effect of 1 mM NOV on DNA single-strand breaks induced by 120 mM EMS for 1 hour in synchronized CHO cells.

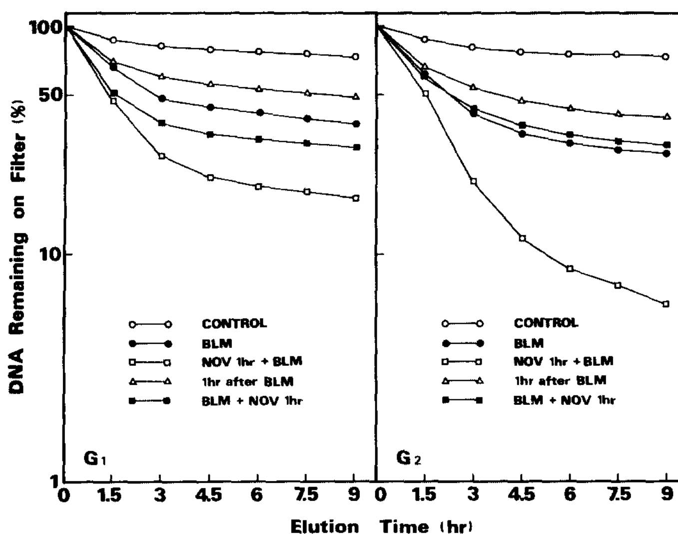

Fig. 3. Effect of 1 mM NOV on DNA single-strand breaks induced by 800 μg/ml BLM for 1 hour in synchronized CHO cells.

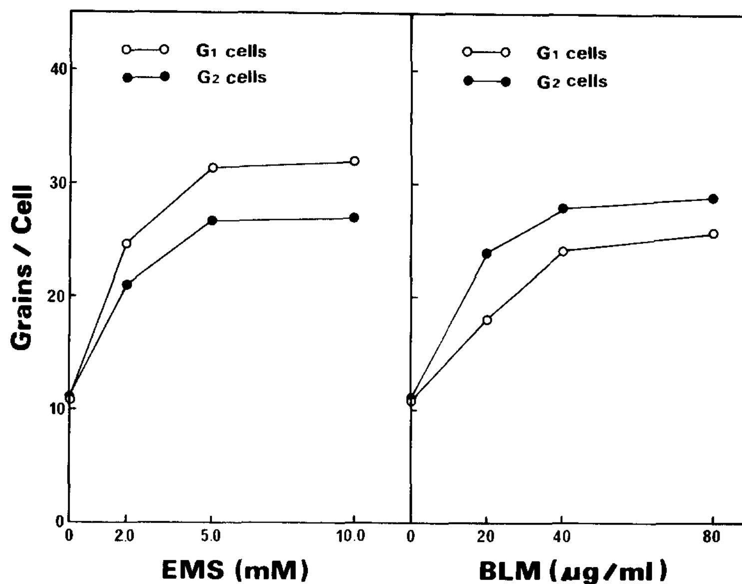

Fig. 4. Effect of unscheduled DNA synthesis induced by EMS or BLM for 1 hour in synchronized CHO cells.

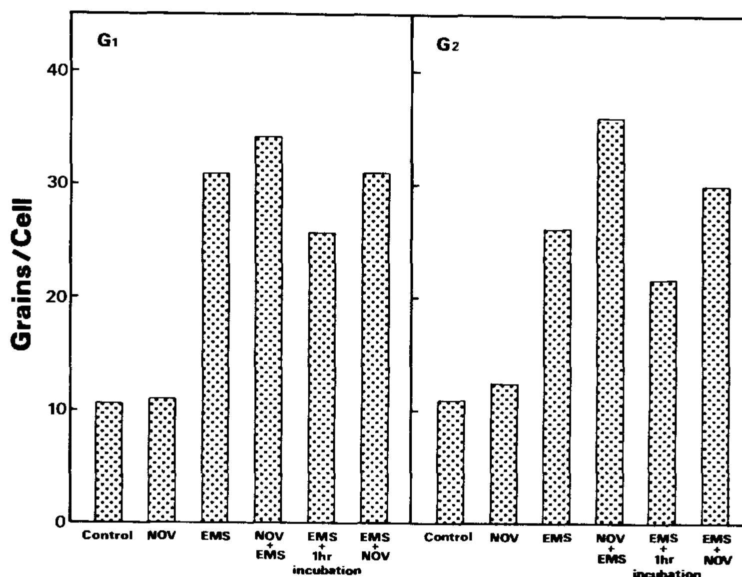

Fig. 5. Effect of 10 μM NOV on unscheduled DNA synthesis induced by 5 mM EMS for 1 hour in synchronized CHO cells.

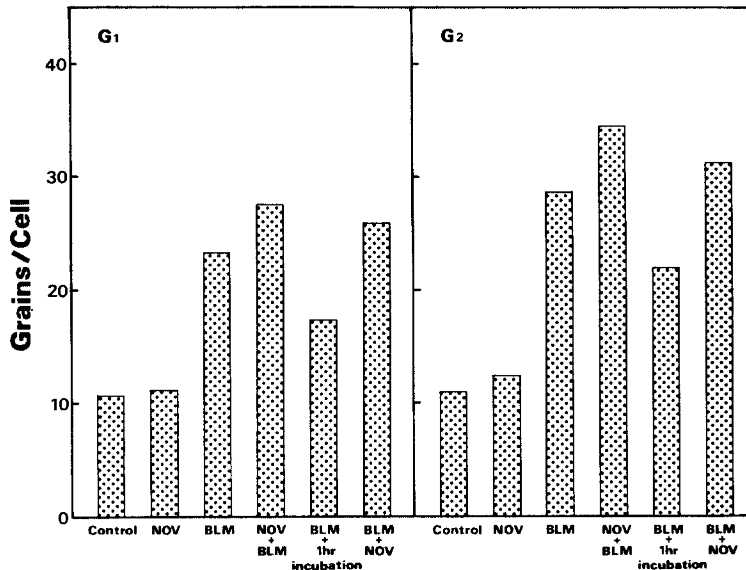

Fig. 6. Effect of 10 μM NOV on unscheduled DNA synthesis induced by 40 μg/ml BLM for 1 hour in synchronized CHO cells.

in G_1 and G_2 phases. And the group pre-treated with NOV is inhibited much more than the group post-treated with NOV in EMS- or BLM-induced unscheduled DNA synthesis.

DISCUSSION

Different sensitivities to radiation and chemical carcinogens during the cell cycle of cultured mammalian cells provide a useful system for studies on the relationships among DNA damage and its repair, survival of cell and mutagenesis (Watanabe and Horikawa, 1980).

Liu *et al.*, (1980) reported that novobiocin (NOV) inhibited a type II eukaryotic DNA topoisomerase: this class of enzyme seems a likely target for the effect of NOV on DNA repair, since the DNA relaxation step is inhibited. And also NOV appears to inhibit the incision step of repair, but it is nuclear whether this is through its action on DNA topoisomerase (Downes *et al.*, 1985; Johnson *et al.*, 1982; Mattem and Scudiero, 1981) or on the activities possibly associated with inhibition of ATP-dependent reactions (Downes *et al.*, 1985; Edenberg, 1980; Johnson *et al.*, 1982; Meechan *et al.*, 1984). In spite of uncertainties about the precise mode of action of this inhibitor, available evidence suggests that NOV acts at earlier step of DNA repair, and it is accompanied by a relaxation of the secondary structure of the DNA (Mattern *et al.*, 1982; Cleaver, 1987).

Tricoli *et al.* (1985) reported that DNA topoisomerase II specific activity showed no detectable change during G_1, S, and M phases of the cell cycle in mouse embryo fibroblasts cells. Hsiang *et al.* (1988) demonstrated that the topoisomerase II level in HeLa cells synchronized by a double thymidine block remained relatively constant throughout the late G_1, S, G_2, and M phases of the cell cycle. Present data also represents that pre-treatment with NOV inhibits the repair of DNA damage induced by EMS or BLM in G_1 and G_2 phases of CHO cells, which suggests that topoisomerase II level in CHO cells synchronized is constant in G_1 and G_2 phases. Cozzarelli (1980) indicated that NOV inhibited topoisomerase and polymerase α, the polymerase thought to be involved in DNA repair. Clarkson and Mitchell (1983) reported that NOV inhibited a form of α polymerase, which was responsible for excision and resynthesis during DNA repair and some other NOV-sensitive polymerase was responsible for excision and resynthesis. Meechan *et al.* (1984) reported that NOV inhibited polymerization and ligation of DNA in vitro. The results of this paper demonstrated that post-treatment with NOV inhibited EMS- or BLM-induced DNA repair synthesis in G_1 and G_2 phases in CHO cells.

Considering above others' and our results obtained, it may be suggested that there is differential sensitivity in relation to cell cycle in cytotoxicity and DNA repair process according to used chemical, and that DNA topoisomerase II participates in earlier step of DNA repair induced by EMS or BLM.

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환경성 유해요인이 유전물질과 세포활성에 미치는 영향
Ⅳ. 동시화된 CHO세포에서 DNA 회복합성에 미치는 Novobiocin의 저해효과

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Chinese hamster ovary (CHO)세포의 세포주기에 따라 ethyl methanesulfonate (EMS) 혹은 bleomycin (BLM)에 의해 유발된 DNA 회복합성에 미치는 DNA topoisomerase II의 저해제인 novobiocin (NOV)의 영향을 조사하기 위하여 세포생존율, 알칼리 유출법, 비주기성 DNA 합성법으로 조사한 결과는 다음과 같다. EMS는 G_1기에서, BLM은 G_2기와 S기에 높은 치사율을 보였고, EMS는 G_1기에서 DNA 상해정도가 심한 반면 BLM은 G_2기에서 상해효과가 높았다. NOV 전, 후처리군은 G_1과 G_2기에서 EMS나 BLM에 의해 유발된 DNA 회복합성을 저해하였으며, 특히 전처리군이 후처리군에 비해 더 높은 저해효과를 나타내었다. 이런 결과들은 CHO 세포는 세포주기와 처리된 돌연변이원의 종류에 따라 세포치사율과 DNA 회복합성이 서로 다른 감수성을 보였고, DNA topoisomerase II의 참여는 아마도 DNA 회복합성과정중 초기단계로 추측된다.