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Nitric oxide blocks the cell cycle of mouse macrophage-like cells in the early G_2+M phase

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Abstract

The effects of nitric oxide produced by macrophage-like cells (Mml) on the cell cycle were investigated. Mml cells lost proliferative activity in the presence of interleukin-6 (IL-6) and a subpopulation accumulated in the G_2+M phase. This level increased in proportion to the incubation time. The DNA content of the cells was slightly lower than that of Mml cells treated with vinbrastine or demecolcine, drugs which block the cell cycle in the M phase. The peak of the early G_2+M phase was reduced by treatment with N^G-mono-methyl-L-arginine. However, after treatment with exogenous nitric oxide or sodium nitroprusside, the G_0/G_1 phase increased, but the early-G_2+M and the S phase decreased. The flow cytometry pattern in IL-6-treated Mml was the same as that of cytochalasin B-treated Mml. These data suggest that endogenous nitric oxide affects the microfilament system of IL-6-treated Mml cells and blocks the cell cycle in the early G_2+M phase.

Key words: Cell cycle; Nitric oxide; Interleukin-6; Macrophage like cell; Microfilament

1. Introduction

Cell growth, differentiation and apoptosis (programmed cell death) in blood cells are controlled by many factors [1]. In the last few years, the cell cycle of blood cells has gradually become clear. The majority of multipotent stem cells and terminally differentiated blood cells arrest in the G_0/G_1 phase [2]. On the other hand, 20–40% of committed progenitors of granulocyte-macrophages and erythrocytes progress to the S phase of the cell cycle [2]. Similarly, the cell cycle of mouse myeloid leukemia cells (M1), the parental strain of Mml cells, is the same as that of committed progenitors and the cells are arrested in the G_0/G_1 phase by treatment with IL-6 [3].

Nitric oxide (NO) is a molecule of current interest owing to its multiple biological functions [4]. In the immune system, NO and other nitrogen oxides produced by activated macrophages have been shown to be a cytostatic and highly cytotoxic agent against tumor cells and parasites [5,6]. Cell death occurs in most animal tissues at some stage of their development and differentiation. NO-producing fibroblasts commit suicide by depression of mitochondrial respiration [7]. Similar phenomena have been reported in NO-producing macrophages [8,9]. However, recently we observed that Mml cells treated with IL-6 were not autocytotoxic by nitric oxide production and reversibly proliferate on removal of IL-6 from the culture medium (manuscript submitted). In the present report, we show that IL-6-treated Mml cells produce nitric oxide and accumulate in the early G_2+M phase of the cell cycle.

2. Materials and methods

2.1. Materials

Reagents and their sources were as follows. (Sigma) N^G-methyl-L-arginine (L-NMMA); (Calbiochem), vinblastine; demecolcine, trichostatin A and cytochalasin B (Wako Pure Chemical Co. Ltd.); recombinant human IL-6 was prepared as described previously [10].

2.2. Cell line and culture

WEHI-3B was purchased from the Institute for Fermentation, Osaka. The Mml cells established by Maeda from M1 cells [11] were cultured as described previously [12]. Mml cells ($5–10 \times 10^6$ cells/10 cm dish) were cultured with IL-6 (100 U/ml) for 1–3 days in the presence or absence of L-NMMA and drugs.

2.3. Flow cytometry

Cells (approximately 2×10^6 cells) were harvested at various times, then washed with PBS, fixed with 70% ethanol for 30 min at 4°C and stained with propidium iodide containing RNase, using a cell cycle staining kit (Boehringer Mannheim). Flow cytometric analysis was carried out on a FACSstar plus. At least 10^4 cells were measured using a standard analysis protocol with a flow rate of 50 μl/min. Histograms were stored and files reanalyzed using the CELL-FIT program (Becton Dickinson).

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3. Results and discussion

Mml cells activated with IL-6 for 2 days ceased DNA synthesis and produced nitric oxide. The cell cycle of Mml cells treated with IL-6 was examined by staining their DNA. As shown in Fig. 1A, approximately 36.6% of the cells were in S phase and 14.2% were in the G_2+M phase. These percentages are similar to those of populations of committed macrophage progenitors [2]. On the other hand, Mml cells treated with IL-6 for 2 and 3 days exhibited a significant increase in the percentage of G_2+M phase cells (Fig. 1). The nitrite concentration of culture medium in IL-6-treated Mml cells for 2 and 3 days was 80.2 and 110.5 (nmol/10^6 cells), respectively. To establish where in the G_1 and G_2 phase the cells arrested, Mml cells were treated with trichostatin A, a histone deacetylase inhibitor which blocks the cell cycle at the G_1 and G_2 phases [13]. As shown in Fig. 1, 66.3% of Mml cells treated with trichostatin A arrested in the G_1 phase and the percentage of G_2+M phase cells was clearly reduced. As WEHI-3B cells are also induced to differentiate by IL-6 [14], the cell cycle of IL-6-treated cells was investigated. As shown in Fig. 1, 87.5% of the cells were arrested in the G_1 phase. Resnitzky reported that the majority of M_1 cells were arrested in the G_1 phase on treatment with IL-6 [3]. M1 and WEHI-3B cells were differentiated, but could not be induced to produce nitric oxide by treatment with IL-6 ([12] and data not shown). These results indicate that the main checkpoint of the cell cycle in Mml cells exists in the G_1 phase and that nitric oxide abrogates this G_1 checkpoint.

To investigate this, Mml cells were treated with IL-6 in the presence of L-NMMA (competitive inhibitor of nitric oxide production). As shown in Fig. 2, percentages of G_2+M phase cells gradually decreased with the corresponding increases in addition of L-NMMA. The nitrite concentration of culture medium of IL-6-treated Mml cells in the presence of 10 μM, 50 μM, 100 μM and 500 μM L-NMMA were 101.7, 89.5, 58.9 and 40.3 (nmol/10^6 cells), respectively. These data suggest that the concentration of nitric oxide correlates with the percentage of G_2+M phase cells. On the other hand, the percentage of G_0/G_1 phase cells was markedly increased with treatment of sodium nitroprusside alone (Fig. 3). It has been reported that nitric oxide inhibits DNA synthesis by inactivation of ribonucleotide reductase [15,16]. Therefore, this result suggests that SNP alone inhibits DNA synthesis and blocks the cell cycle prior to the S phase.

DNA content of the major peak of G_2+M phase-arrested IL-6-treated Mml was 1.76–1.88 times that in the cells arrested in the G_0/G_1 phase. To identify the checkpoint of the cell cycle, Mml cells were incubated

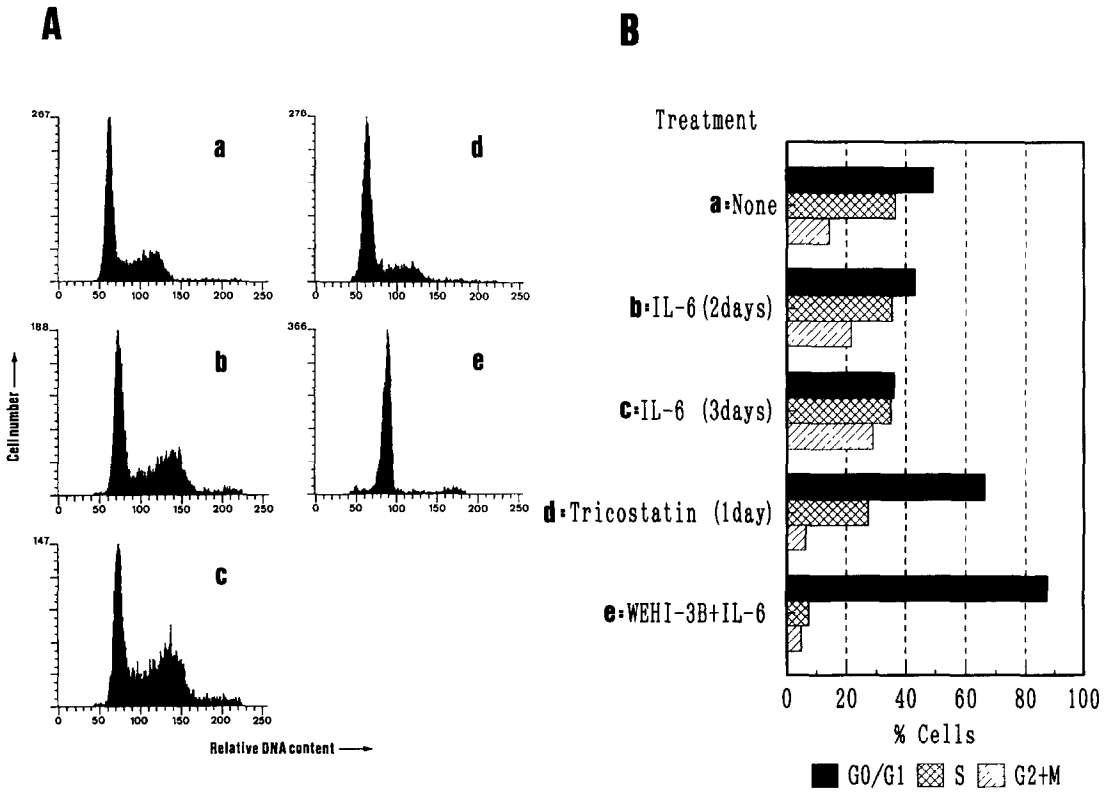

Fig. 1. Cell cycle changes in Mml cells after treatment with IL-6. (A) Flow cytometry charts of Mml cells cultured in the absence (a) or in the presence of IL-6 (100 U/ml) for 2 days (b) and 3 days (c), and trichostatin A (0.5 μg/ml) for 1 day (d). (e) Flow cytometry chart of WEHI-3b cells cultured with IL-6 (100 U/ml) for 3 days. Cells were stained with PI and analyzed by FACSstar plus as described in section 2. (B) Quantitative assessment of the percentages of Mml cells remaining in the G_0/G_1 phase, the S phase, and the G_2+M phase, evaluated as illustrated in (A).

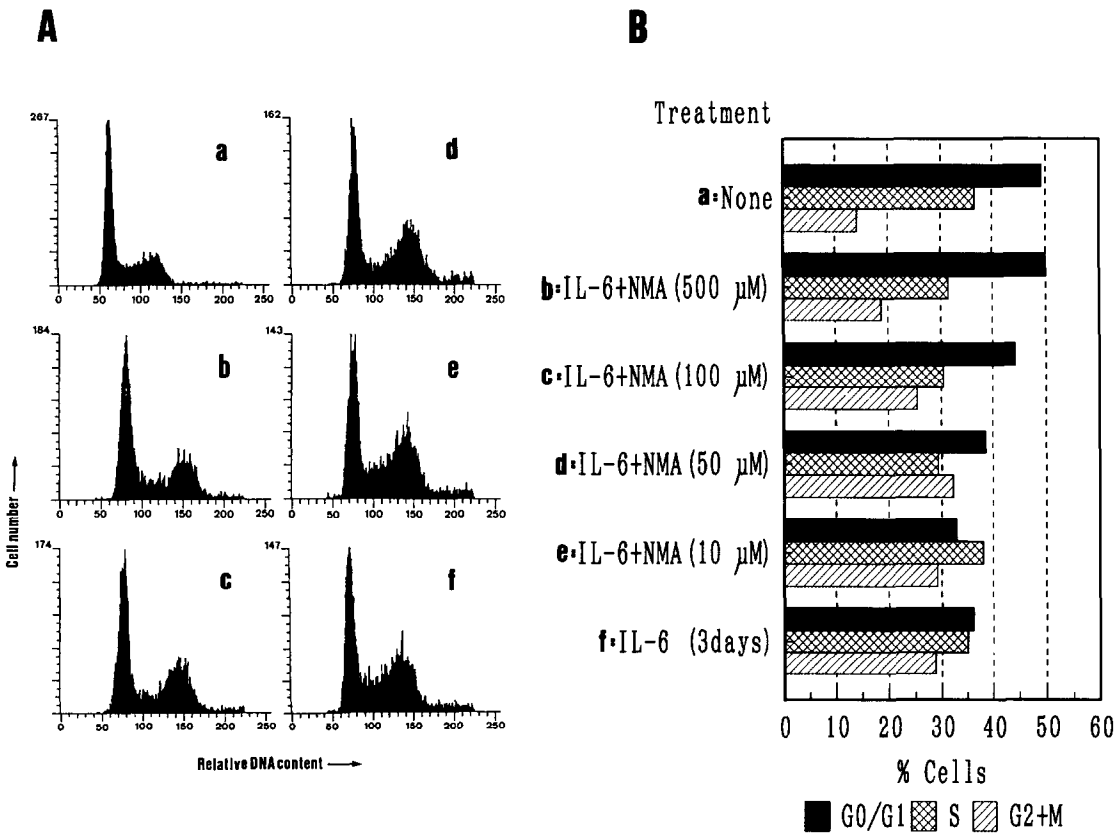

Fig. 2. Effects of L-NMMA on cell cycle in IL-6-treated Mm1 cells. (A) Flow cytometry chart of Mm1 cells treated with IL-6 (100 U/ml) for 3 days in the absence (a) or in the presence of 500 μM (b), 100 μM (c), 50 μM (d) and 10 μM (e) of L-NMMA. (f) Flow cytometry chart of mM1 cells cultured in the absence of IL-6 for 2 days. The cells were stained with PI and analyzed by FACSstar plus as described in section 2. (B) Quantitative assessment of the percentages of Mm1 cells remaining in the G_0/G_1 phase, the S phase and the G_2+M phase, evaluated as illustrated in (A).

with vinblastine, demecolcine or cytochalasin B in the presence or absence of IL-6. As shown in Fig. 4, demecolcine and vinblastine completely inhibited the cell cycle in the G_2+M phase and the DNA content of those cells was approximately double that of G_0/G_1 phase cells. Interestingly, the cell cycle of Mm1 cells treated with cytochalasin B was the same as that of IL-6-treated Mm1 cells (Fig. 4). In the presence of IL-6 (100 U/ml), the cell cycle was arrested in the early G_2+M phase after treatment with demecolcine and vinblastine (Fig. 4). It has been reported that nitric oxide activates guanylate cyclase, increases the level of intracellular c-GMP and subsequently relaxes venous smooth muscle. The mechanism is thought to be that the myosin light chain (MLC) is phosphorylated by MLC-kinase in a calmodulin-dependent manner, inducing the dissociation of myosin and actin filaments [17,18]. In addition, it has been shown in vitro that actin is ADP-ribosylated by nitric oxide and the ADP-ribosylated actin is unable to polymerize [19,20]. These observations and the data in the present report suggest that endogenous nitric oxide acts on actin filaments and blocks the cell cycle in the early G_2+M phase. However, the effect of nitric oxide alone on the cell cycle is not sufficient without a signal from IL-6.

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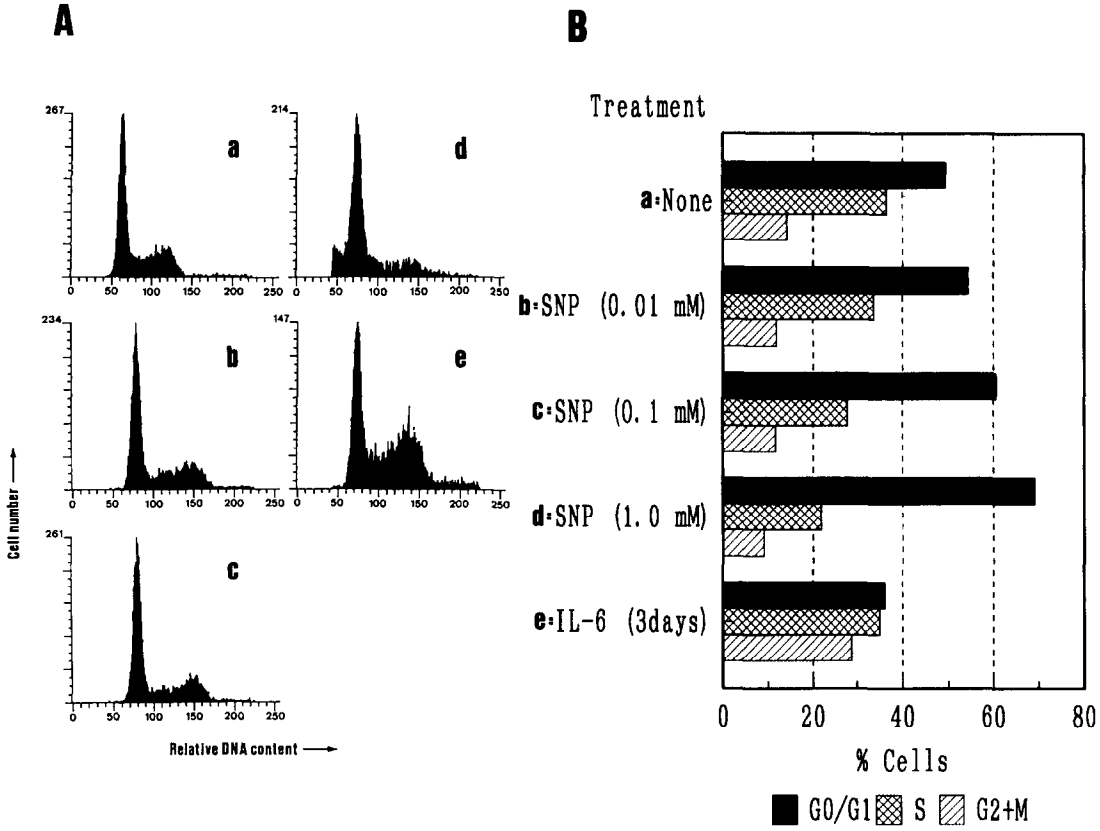

Fig. 3. Effects of SNP on cell cycle in Mm1 cells. (A) Flow cytometry chart of Mm1 cells cultured in the absence (a) or in the presence of 0.01 mM (b), 0.1 mM (c) and 1.0 mM (d) of SNP. The cells were stained with PI and analyzed by FACSstar plus as described in section 2. (B) Quantitative assessment of the percentages of Mm1 cells remaining in the G_0/G_1 phase, the S phase and the G_2+M phase, evaluated as illustrated in (A).

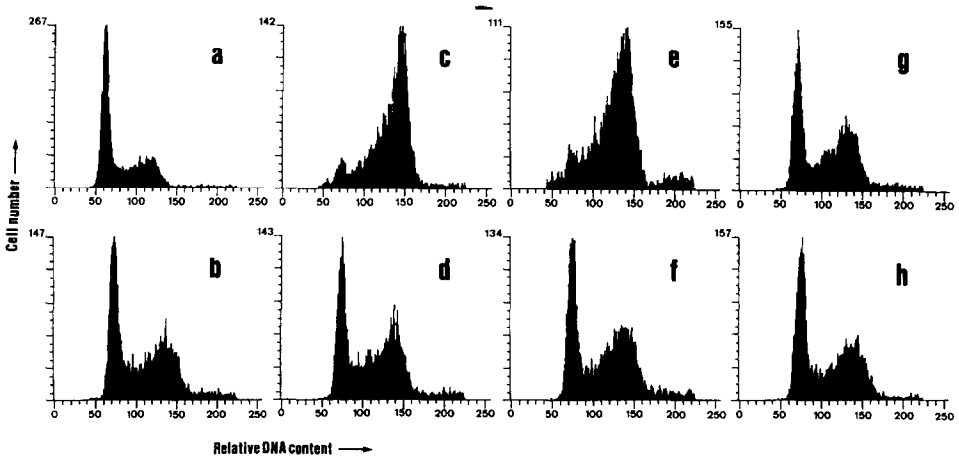

Fig. 4. Identification of the cell cycle checkpoint in IL-6-treated Mm1 cells. Mm1 cells cultured in the absence (a,b) or in the presence of demecolcine (0.05 μg/ml) (c,d), vinblastine (10^{-7} M) (e,f), and cytochalasin B (10^{-7} M) (g,h) for 3 days without (a,c,e,g) or with IL-6 (100 U/ml) (b,d,f,h). The cells were stained with PI and analyzed by FACSstar plus as described in section 2.

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