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Isothiocyanates inhibit cell cycle progression of HeLa cells at G_2/M phase

Teiko Hasegawa, Hoyoku Nishino and Akio Iwashima

Department of Biochemistry, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji Kamigyo-ku, Kyoto 602, Japan. Tel: (+81) 75-251-5315. Fax: (+81) 75-213-2746.

To analyze the inhibitory effects of isothiocyanates on the growth of human cancer cells, the effects of these compounds on the cell cycle progression of HeLa cells were studied by flow cytometry. The accumulation of cells at G_2/M phase was observed at 16 h after treatment with 10 μM allyl isothiocyanate, 2.5 μM benzyl or phenethyl isothiocyanate, at which concentrations the cell growth was inhibited to 41–79% of control. These results suggest that isothiocyanates delay the cell cycle progression of HeLa cells, leading to inhibition of cell growth.

Key words: G_2/M phase arrest, growth inhibitory effect, isothiocyanate.

Introduction

Isothiocyanates are known to be pungent compounds in *Wasabia japonica*, horse radish (*Brassica hirta* Moench.), brown mustard (*Brassica nigra* Koch.), leaf mustard (*Brassica juncea*) and cress (Holland mustard), which are used as spices. They have been shown to block chemical carcinogenesis; 1-naphthyl isothiocyanate suppressed hepatoma formation induced by diethylnitrosamine in rats,[1] and benzyl isothiocyanate blocked tumor formation by diethylnitosamine or benzo[*a*]pyrene in lung and forestomach in mice.[2,3]

Furthermore, it has been reported that the uptake of cruciferous vegetables reduces the risk of cancer development; as one of the possible mechanisms of this protection, the induction by isothiocyanates of phase II enzymes which are involved in the metabolism of carcinogens has been proposed.[1,4,5]

It is therefore of interest to study whether isothiocyanates have anti-cancer activity besides anti-carcinogenic activity. In this paper the effect of isothiocyanates on the proliferation of HeLa cells was investigated.

Correspondence to T Hasegawa

Materials and methods

Chemicals

Allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PITC) were obtained from Nakalai tesque (Kyoto, Japan) and stored in sealed containers at 4°C. They were dissolved in dimethylsulfoxide (DMSO) and diluted to the appropriate concentration just before each experiment. TN-16 [3-(1-anilino-ethylidene)-5-benzylphenolidine-2,4-dione] and aphidicolin were obtained from Wako Pure Chemicals, (Osaka, Japan). Propidium iodide was purchased from Calbiochem (La Jolla, CA) and RNase was obtained from Sigma (St Louis, MO).

Cell culture

HeLa cells were cultured at 37°C in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO_2 in air. The cells were subcultured at a density of 8×10^4 cells/2 ml medium in 35 mm diameter Petri dish for the growth experiment and at a density of 1.5×10^6 cells/6 ml in 25 cm^2 flasks for cell cycle analysis.

At 48 h after the inoculation of cells, isothiocyanates were added to the culture medium and then the incubation was continued for a designated period. A culture treated with DMSO alone at the same concentration (0.2%) served as the control.

Determination of the number of viable cells

Cells were trypsinized and counted. Viability of cells was determined by the Trypan blue exclusion method.

Flow cytometric analysis

Cells were removed at the indicated time from flasks by trypsinization. After washing twice with phosphate-buffered saline (PBS), 1×10^6 cells were centrifuged and suspended in PBS containing 0.1% Triton X-100. Then the suspension was filtered through 50 μm nylon mesh and incubated with 0.1% RNase and 50 μg/ml propidium iodide for 15 min. DNA content in stained cells was analyzed with FACScan (Becton-Dickinson, Mountain View, CA). A suspension of 1×10^4 cells was analyzed for each DNA histogram. The percentage and cell number in each phase were determined according to the R fit program in the FACScan.

Synchronization of cell culture

After 48 h of inoculation, 1 μg/ml of aphidicolin was added to the culture medium and the culture was continued for 24 h at 37°C to obtain synchronized cells in G_1 and S phases. Cells synchronized in M phase were obtained by treatment with 0.5 μM TN-16 for 24 h.

Statistical analysis

The Student's *t*-test was used to determine the significance of difference between experimental and control groups. Each point represents the mean of three experiments in triplicate with the standard deviation below 10%.

Results

Effect of isothiocyanates on the proliferation of HeLa cells

Figure 1 shows the effect of AITC on the growth of HeLa cells. AITC at 2, 5, 10, 15 and 20 μM decreased the growth rate of HeLa cells to 90, 84,

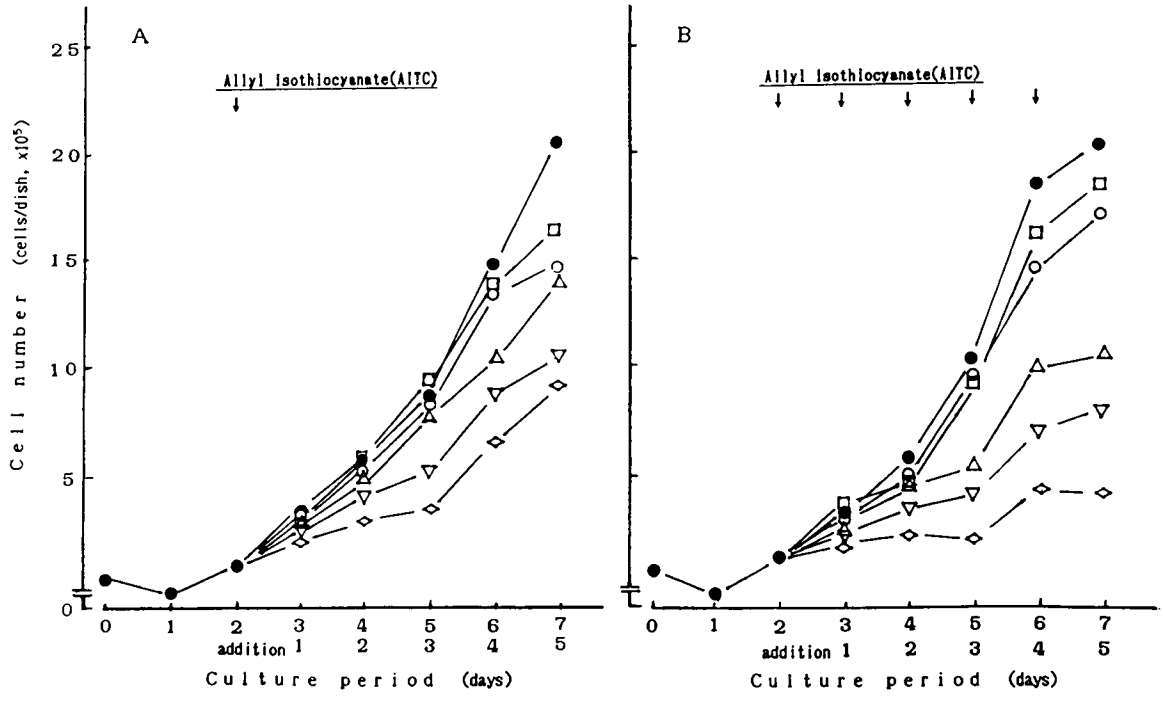

Figure 1. Effect of AITC on the proliferation of HeLa cells. HeLa cells were inoculated at a density of 8×10^4 cells/dish. After 48 h, AITC at concentrations of 2 μM (□), 5 μM (○), 10 μM (△), 15 μM (▽) and 20 μM (◇) (or 0.2% DMSO (●) as the control) was added to culture medium, as indicated by an arrow. In experiment (A), medium was not changed, while in experiment (B), the medium was removed from dishes, and the cells were washed twice with PBS and new medium with freshly prepared AITC added daily. The number of viable cells was counted daily. Each point represents the mean of triplicate experiments.

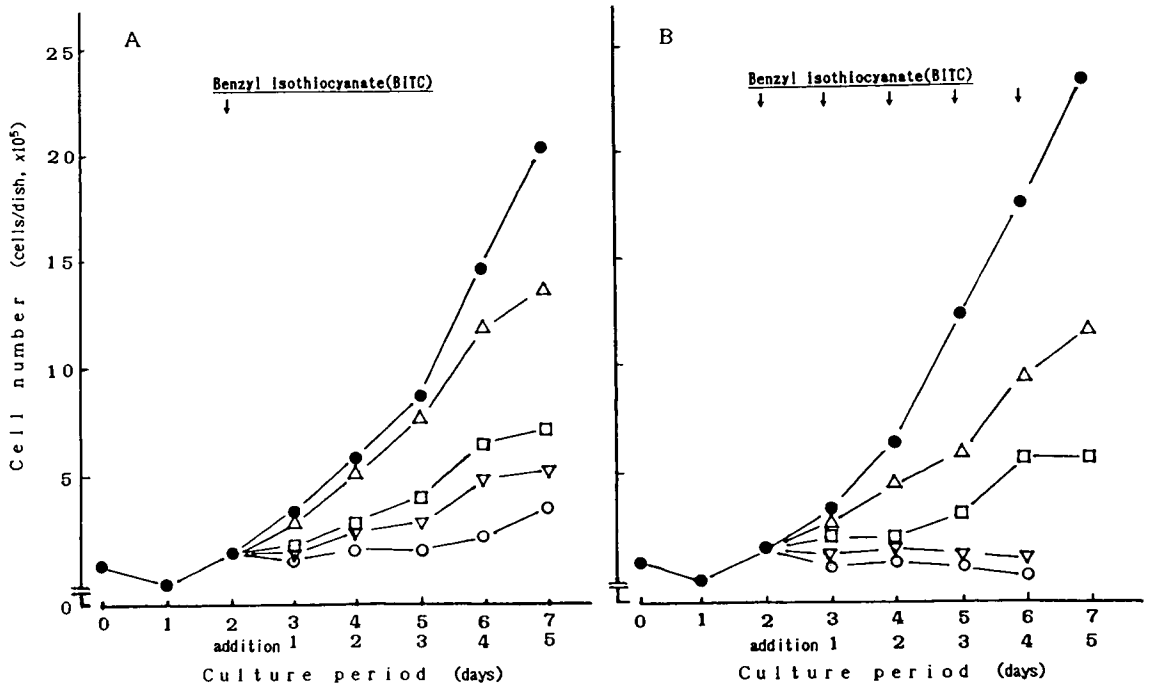

Figure 2. Effect of BITC on the proliferation of HeLa cells. BITC at concentrations of 1 μM (△), 2 μM (□), 2.5 μM (▽) and 5 μM (○) (or 0.2% DMSO (●) as the control) was added to culture medium as indicated by an arrow. The experiments were carried out as described in Figure 1: (A), without medium change; (B), with medium change.

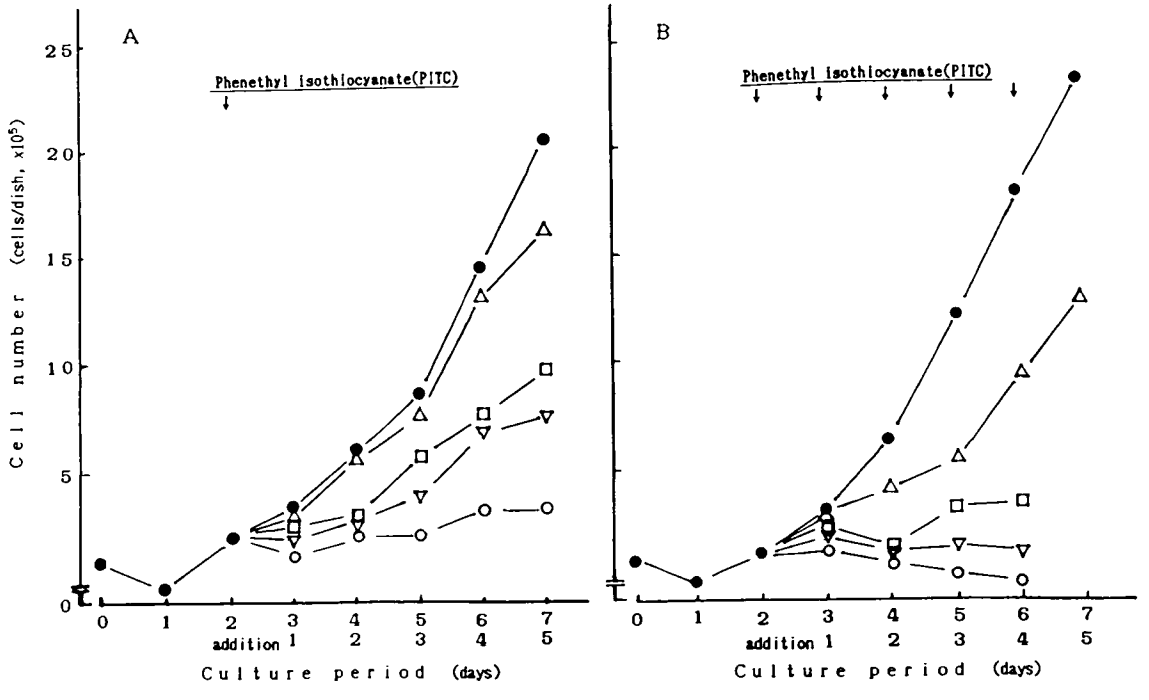

Figure 3. Effect of PITC on the proliferation of HeLa cells. PITC at concentrations of 1 μM (△), 2 μM (□), 2.5 μM (▽) and 5 μM (○) (or 0.2% DMSO (●) as the control) was added to culture medium, as indicated by an arrow. The experiments were carried out as described in Figure 1: (A), without medium change; (B), with medium change.

67, 56 and 43% of the control at day 4 after the drug treatment, respectively (Figure 1A). Thus, AITC inhibited the growth of HeLa cells in a dose-dependent manner and the IC_{50} was about 15 μM. Since AITC is a volatile compound, the amount of AITC in culture medium might be decreased during the experiments. To test this possibility, cells were washed twice with PBS and the medium with freshly prepared AITC at the defined concentration was added to the culture every 24 h. Under these experimental conditions, the number of cells were decreased to about 86, 78, 53, 37 and 23% of control at day 4 after the treatment with 2, 5, 10, 15 and 20 μM AITC (Figure 1B), respectively. Thus, some portion of AITC seems to be lost during the period of culture.

Figures 2 and 3 show the alteration of growth curves of HeLa cells in the presence of BITC or PITC. Both BITC and PITC suppressed the growth of HeLa cells in a dose-dependent manner. IC_{50} values at day 4 after the treatment with BITC or PITC once were 1.9 and 2.5 μM, respectively (Figures 2A and 3A). IC_{50} values at day 4 in the case of fresh BITC or PITC treatment every 24 h were 1.0 and 1.2 μM, respectively (Figures 2B and 3B). Thus, the growth inhibitory effects of BITC and PITC on HeLa cells were stronger than that of AITC. In any case, these results clearly show that isothiocyanates have an inhibitory effect on the proliferation of HeLa cells.

Effects of isothiocyanates on cell cycle progression

To analyze the inhibitory effects of isothiocyanates on the proliferation of HeLa cells, the alteration of cell cycle progression was examined by flow cytometry. HeLa cells were treated with AITC, BITC or PITC at various concentrations for a designated period. DNA contents of cultured cells were measured by flow cytometric analysis. Figure 4 shows the DNA histograms of the cells treated with various concentrations of isothiocyanates for

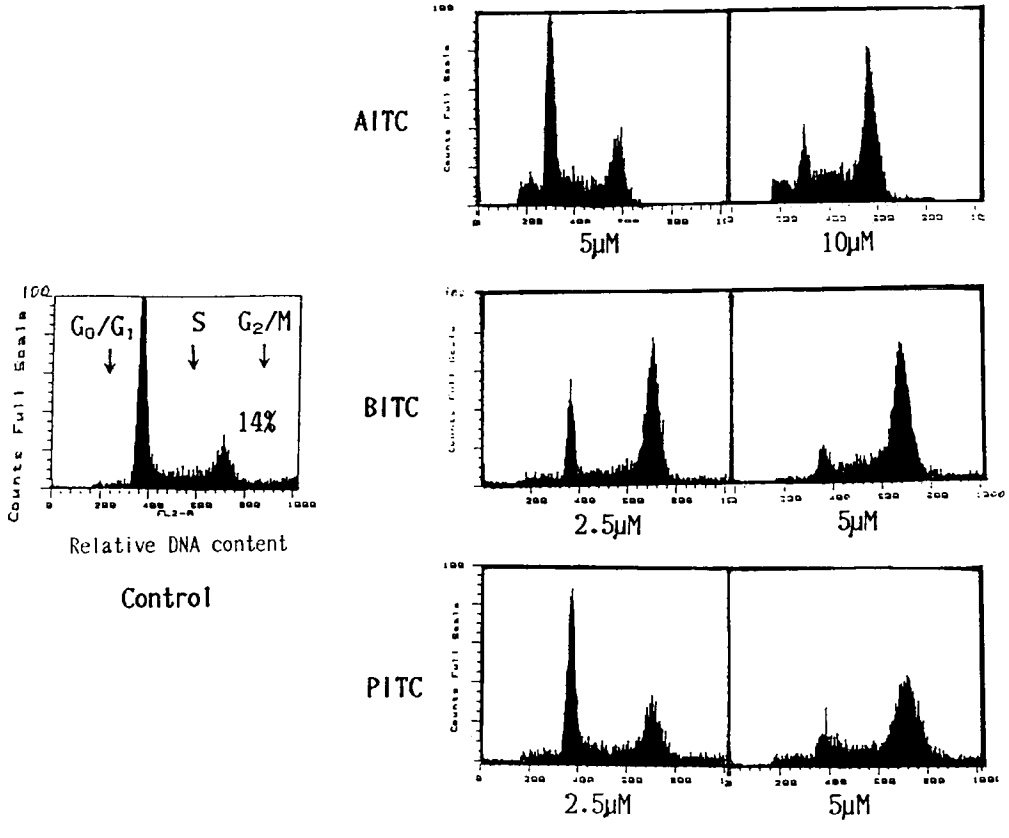

Figure 4. DNA histograms of HeLa cells treated with various concentrations of AITC, BITC or PITC. Effects of AITC (5 and 10 μM), BITC (2.5 and 5 μM), PITC (2.5 and 5 μM) or DMSO as control were analyzed by flow cytometry using the *R* fit program at 16 h after addition of these compounds. Triplicate experiments were repeated at least four times. Representative data are shown.

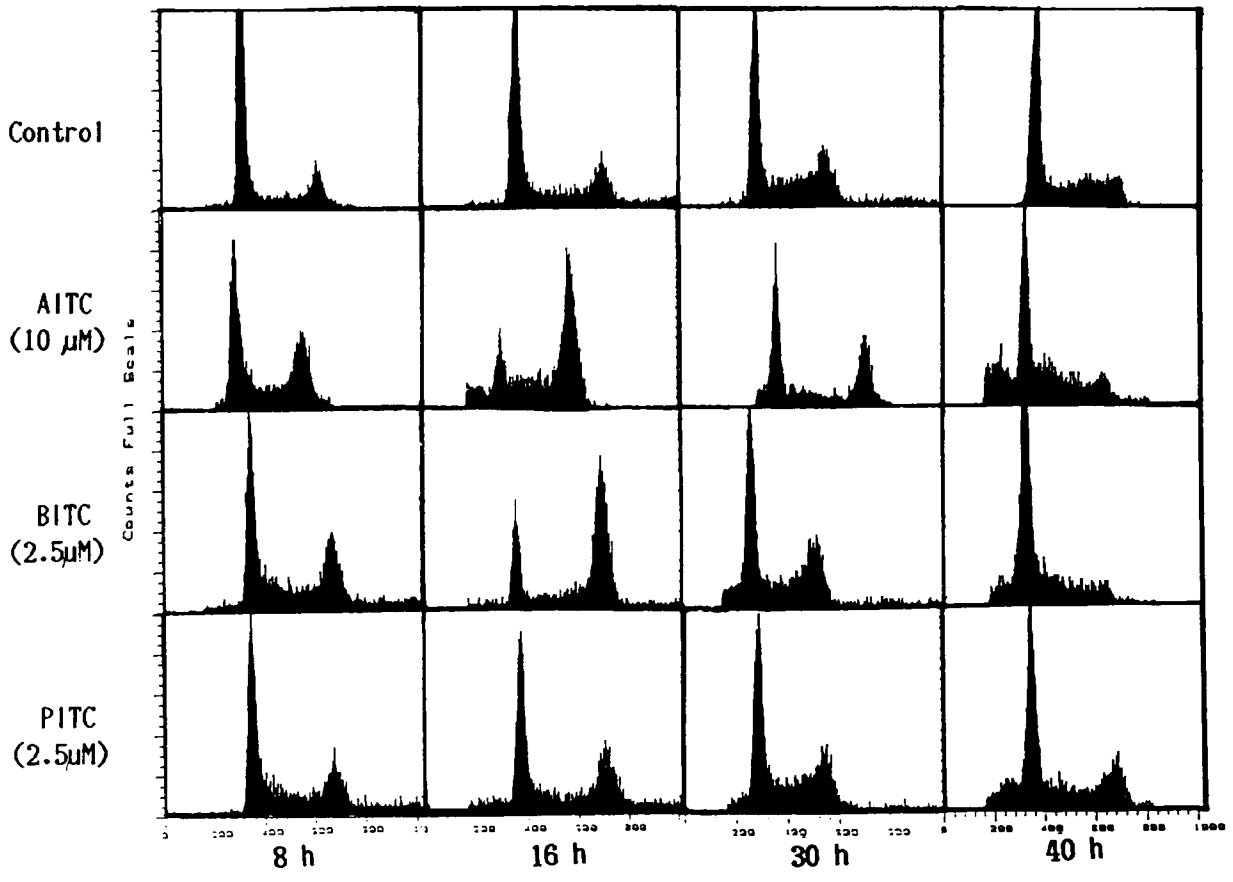

Figure 5. DNA histograms of HeLa cells treated with AITC, BITC or PITC for various periods. The time course of the cell cycle progression was compared in cells treated with either AITC (10 μM), BITC (2.5 μM), PITC (2.5 μM) or DMSO as control. Triplicate experiments were repeated at least three times. Representative data are shown.

16 h. The percentage of cells in G_2/M phase were higher and in G_1 phase were lower than that of the control. The predominant accumulation in G_2/M phase and conversely the low levels in G_1 phase were observed with 10 μM AITC, 5 μM BITC and 5 μM PITC. We further examined the effects of 10 μM AITC, 2.5 μM BITC and 2.5 μM PITC at various incubation times after the addition of each isothiocyanate. As shown in Figure 5, the maximum accumulation in G_2 M phase was observed at 16 h after treatment with 10 μM AITC, 2.5 μM BITC or 2.5 μM PITC.

The accumulation of G_2 M phase cells was confirmed in the synchronized cells prepared by the addition of aphidicolin or TN-16.

As shown in Figure 6, cells treated with aphidicolin (1 μg ml) for 24 h were synchronized in G_1 or S phase, and the progression of the cell cycle returned to the original pattern at 20 h after washing-out of aphidicolin. In the presence of 20 μM AITC, 2.5 μM BITC or 2.5 μM PITC, progression of the cell cycle was retarded at G_2/M phase. As shown in Figure 7, cells synchronized in M phase by 0.5 μM TN-16 entered into G_1 phase at 20 h after washing-out of TN-16. In the presence of 10 μM AITC, 2.5 μM BITC or 2.5 μM PITC, progression of the cell cycle from G_2/M phase to G_1 phase was obviously retarded. These results indicate that AITC, BITC and PITC had similar effects on the cell cycle progression of HeLa cells, although the effects of BITC or PITC were stronger than that of AITC. In conclusion, isothiocyanates seem to show anti-proliferative effects through the induction of cell cycle arrest, especially at G_2 M phase.

Discussion

This is the first report which demonstrates the potential of isothiocyanates to suppress the progression of cell cycle at G_2 M phase, resulting

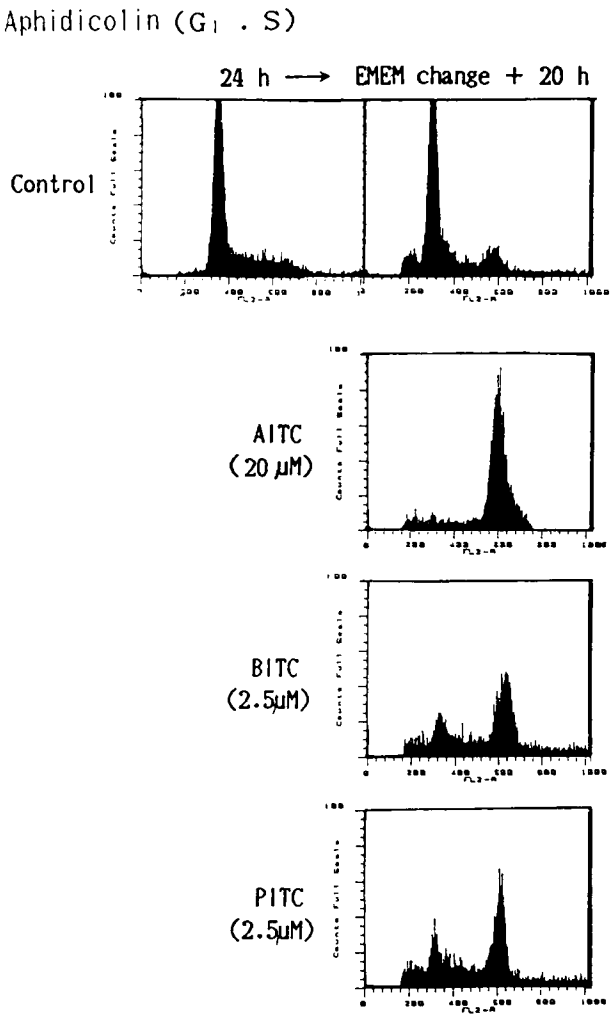

Figure 6. DNA histograms of HeLa cells treated with AITC, BITC or PITC after synchronization by aphidicolin. HeLa cells were synchronized in G_1 and S phases of the cell cycle by aphidicolin, and then treated with AITC (20 μM), BITC (2.5 μM) or PITC (2.5 μM) for 20 h. Triplicate experiments were repeated at least three times. Representative data are shown.

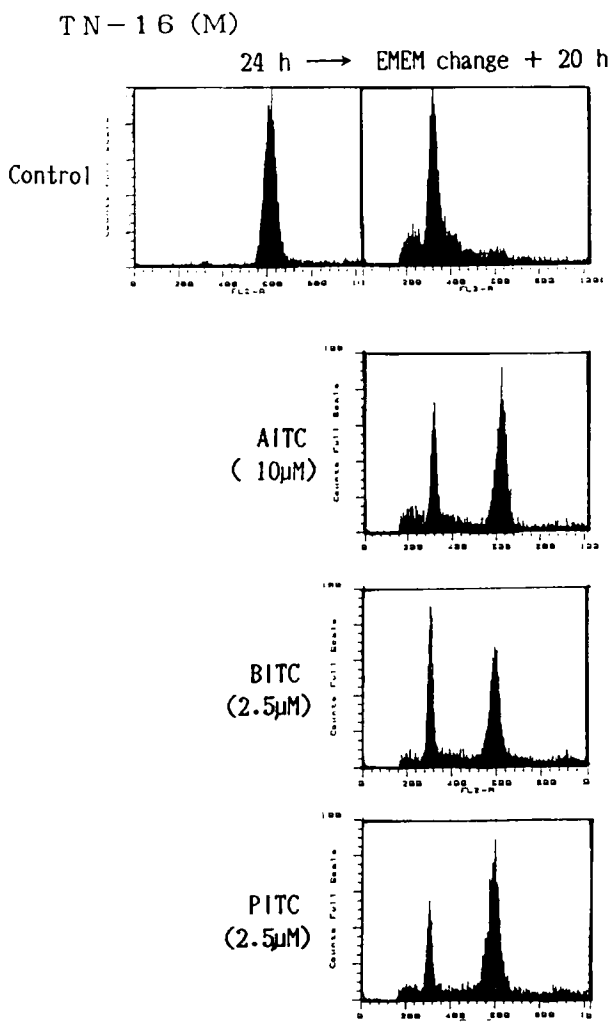

Figure 7. DNA histograms of HeLa cells treated with AITC, BITC or PITC after synchronization by TN-16. HeLa cells were synchronized in M phase of the cell cycle by TN-16 and then treated with AITC (10 μM), BITC (2.5 μM) or PITC (2.5 μM) for 20 h. Triplicate experiments were repeated at least three times. Representative data are shown.

in the inhibition of cell proliferation of human cancer cells.

The most common pungent isothiocyanates, which are produced from their precursors by the action of myrosinase, are AITC (released from sinigrin), and BITC and PITC (released from sinalbin).

These are volatile and relatively unstable compounds, usually converting to thiocarbamate.[6] Therefore, in some experiments, freshly prepared isothiocyanates were added every 24 h. The results suggest that some degradation may occur during the cell culture, resulting in the decrease of anti-tumor potency, which could be overcome by a change of medium to keep the medium containing fresh isothiocyanates.

The mechanism for the induction of the G_2/M arrest is not yet clear. Furthermore, it should be first elucidated whether isothiocyanates induce G_2 arrest or M arrest. Simple analysis by flow cytometry is generally unable to discriminate between G_2 and M arrest. However, using HeLa cells synchronized in M phase by TN-16 we found that isothiocyanates inhibit cell cycle progression at the M phase. G_2 arrest, besides M arrest, may also be induced by isothiocyanates, although there is no way at present to confirm this possibility. Further investigations to elucidate the precise

mechanism of action of isothiocyanates to suppress cell cycle progression at G_2/M phase should be carried out.

As described previously, isothiocyanates not only have anti-proliferative activity but also have been demonstrated to show anti-tumorigenic activity. Some part of the anti-tumorigenic activity of isothiocyanates may be explained by their induction of G_2/M arrest.

Conclusion

The alteration of cell cycle progression by isothiocyanates is closely related to their inhibitory effects on the cell growth of HeLa cells. Isothiocyanate-induced accumulation of G_2/M phase cells results in the delay of cell cycle progression and hence in the suppression of proliferation of human cancer cells.

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