Short communication J. Trad. Med. 20, 168－172, 2003

Extract of *Cassia nomame* has effect on the cell cycle of CHO-K1 cells and suppresses chromosome aberrations induced by Mitomycin C

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(Received March 27, 2003. Accepted May 29, 2003.)

Abstract

Chinese hamster ovary K1 (CHO-K1) cells were pretreated with 2.5 μM Mitomycin C (MMC) for 1 h and incubated with or without the extract of *Cassia nomame* (Sieb.) HONDA in medium for 10-24 h. The frequency of cells with chromosome aberrations was significantly lower with the extract than that without the extract. The result of synchronized culture of cells showed that the extract delayed the first division of MMC-pretreated CHO-K1 cells. Since the extract had no effect on the division of non-treated cells, the suppressing effect of the extract on chromosome aberrations induced by MMC must have a relation to the progression of the cell cycle.

Key words *Cassia nomame*, cell cycle, chromosome aberration, mitomycin C, CHO-K1 cell.

Abbreviations MMC, Mitomycin C; CHO-K1, Chinese hamster ovary K1 cells ; PBS, phosphate buffered saline.

Introduction

Many *Cassia* plants, which are known commonly by the name of "senna", produce chemicals that are expected to influence human physiology or pathology. Senna extracts, e.g. extracts from Tinnevelly senna (*Cassia angustifolia* VAHL) and Egyptian senna (*Cassia acutifolia* DELILE), are used worldwide for the therapy of constipation. Mengs *et al.* have confirmed in the mouse micronucleus assay that the senna extract had no clastogenic activity, and concluded that there was no indication of a genotoxic risk for the therapeutic use of senna as a laxative.[1)]

The aqueous extract of the leaves, stems and pods of *Cassia nomame*, called "Hama-cha", is a conventional beverage in the San'in district of Japan. It is also used as a raw material for a diuretic or antidote in a traditional remedy.

We have investigated the suppressing effect of the extract on clastogenesis of MMC which is an anticancer drug and clastogen. We reported previously that the extract of *C. nomame* lowered the frequency of chromosome aberrations in CHO cells (selected by Sasaki from CHO-K1) treated with 2.5μM MMC and normalized their proliferation rate that was suppressed by MMC.[2)]

In this paper, we report that the *C. nomame* extract influences the progression of the first cell cycle after MMC treatment.

Materials and Methods

Preparation of the extract of C. nomome : The aerial parts of *C. nomame* were collected from Nagaoka, Niigata prefecture, Japan. Dried plants were mixed with hot distilled water 30 times more than their weight, and boiled for 1 h. After cooled, the mixture was strained out through a coffee filter and the decoction was filtered through filter paper (ADVANTEC, No3). The filtrate was concentrated by vacuum distillation until it evaporated to one fifteenth of its initial volume. The concentrate was centrifuged at approximately 560 × g for 30 min and the supernatant was recovered. The resulting extract was stored at 4℃. If a sediment appeared in the extract, the supernatant fraction was used.

Cell line and culture medium : CHO-K1 cells used in this study were obtained from Health Science Research Resources Bank (HSRRB, Japan Health

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Sciences Foundation, Japan). Cells were cultured in 60-mm culture dishes containing 5ml of medium per dish, and were cultured under a humidified atmosphere with 5% CO_2 at 37℃.

The basal medium was composed of Ham's F12 medium (DAIGO, Wako Pure Chemical Industry, Japan) supplemented with 10% fetal bovine serum (GIBCO BRL Inc., U.S.A.). The basal medium containing *C. nomame* at 0.8 %(v/v) was incubated for 30-45 min in the culture condition before use.

Mitomycin C treatment : MMC (MITOMYCIN Kyowa S, azirizino [2',3':3,4] pyrrolo [1,2-α] indole-4,7-dione-6-amino-1,1a,2,8,8a,8b-hexahydro-8- (hydroxy methyl) 8a-methoxy-5-methyl-carbamate) was purchased from Kyowa Hakko Kogyo Co., Ltd. in Japan. CHO-K1 cells were plated at a density of 2.5×10^5 cells/dish. They were incubated for 24 h to grow in logarithm phase. MMC was dissolved in PBS at 200 times as high as the final concentration of 2.5μM in culture medium. Cells were incubated with or without MMC for 1 h before test with the *C. nomame* extract. After the MMC treatment, cells were washed with Hanks' balanced salt solution and incubated with or without the extract.

Assay of chromosome aberrations : After incubation for 11-22 h, cells were treated with 125 μM colchicine for 1 h to collect meta-phase cells. Mitotic preparations were obtained by a conventional air-drying method and were stained with 6 %(v/v) Wright Solution in 1/30 M phosphate buffer (pH 6.8). One hundred well-spread metaphase cells were observed for chromosome aberrations at each sampling time under a microscope. The frequency of aberrant cells was determined by counting metaphase cells with one or more chromosome aberrations.

Selection of cells at mitotic phase : CHO-K1 cells were plated at a density of 5×10^5 Cells/dish. Following 24 h incubation, cells were washed once with the basal medium and incubated for 24 h. Then, the medium was removed, and detachable cells were washed out by pipetting with 2 ml basal medium. After culture with the basal medium for 24 h, dishes were horizontally shaken in 80 strokes per min for 25 min at 37℃. M-phase cells, floating in medium after shaking, were transferred to dishes with the medium.

Culture of cells selected at M phase : Two hours after the selection of mitotic cells, the dishes with cells were divided into two groups, one half group incubated with MMC and another half in the basal medium. After 1 h, medium of the dishes of each sub-group of the two above mentioned groups were replaced by that with or without the *C. nomame* extract.

Observation of colonies under a light microscope : About 120 colonies in 9 randomly selected fields of microscopic view were observed for one dish at intervals of 1 or 2 h to count the number of colonies with each number of cells.

Results

Suppression by the C. nomame extract of clastogenicity of MMC

Figure 1 shows profiles of changes of the frequency of metaphase CHO-K1 cells with chromosome aberrations with time of culture in the presence or absence of the *C. nomame* extract after 1 h of MMC treatment. The cells incubated with the extract showed chromosome aberrations at a lower frequency than those incubated in the basal medium in shorter times after MMC treatment. To test the significance of the difference in the frequencies, we conducted the χ^2-test for 2×2 table. The results indicated that the difference is significant ($p < 0.01$).

When cells were cultured in the basal medium after MMC treatment, the frequency of metaphase cells with chromosome aberrations reached a peak around 15 h after the treatment. On the other hand, when cells were

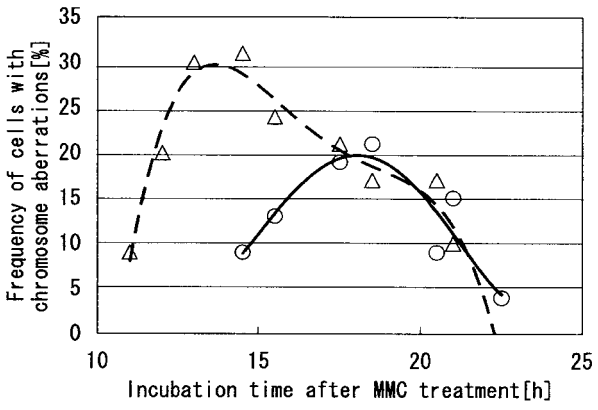

Fig. 1 Suppression of clastogenicity of MMC by *C. nomame* extract. The frequency of cells with chromosome aberrations is shown at serial incubation times after a 1 h treatment with MMC. △ indicates the frequency when cells were incubated in the basal medium after MMC. ○ indicates the frequency when cells were incubated with the extract after MMC. The broken and solid curves show approximate cubic tetranomial curves of △ and ○, respectively.

cultured with the extract, the frequency of metaphase cells with chromosome aberrations reached a peak around 19 h after the MMC treatment. It is possible that the extract has an effect on the cell cycle of cells treated with MMC.

Effect of the extract on the cell cycle

We investigated the effect of the extract on the cell division by using partially synchronized cells. Figure 2 shows the percentage of colonies with different numbers (1-4) of cells at each time after synchronization. Fig.2-1 shows time courses of cell division for the cells cultured in the basal medium. As the proportion of one-cell colonies decreased after 10 h following the synchronization, that of two-cell colonies, in turn, increased with time during 16 h of culture. It is thought that a part of cells began to divide at 10 h after synchronization, all cells by 15 h after synchronization. Two-cell colonies observed within 10 h after synchronization were considered as synchronized in the early M-phase at the time of the selection, and they divided after 15 h into three- or four-cell colonies. These findings suggest that cells would take about 13 h to divide.

Fig.2-2 shows the result of cells cultured in the presence of the extract without MMC pretreatment. The ratio of two-cell colonies reached 50% in 12-15 h after synchronization. Colonies with three or four cells emerged around 15 h after synchronization. These findings suggest that the average division cycle length is 12-15 h. Fig.2-1 and Fig.2-2 showed that the extract has no effect on cell cycle of MMC-nontreated cells.

Fig.2-3 shows the result of the experiment in which cells were pretreated with MMC and cultured in the basal medium. The ratio of two-cell colonies reached 50% at 15 h and was maintained until 18 h after the synchronization. Fig.2-4 shows the result of the experiment in which MMC-pretreated cells were cultured in the

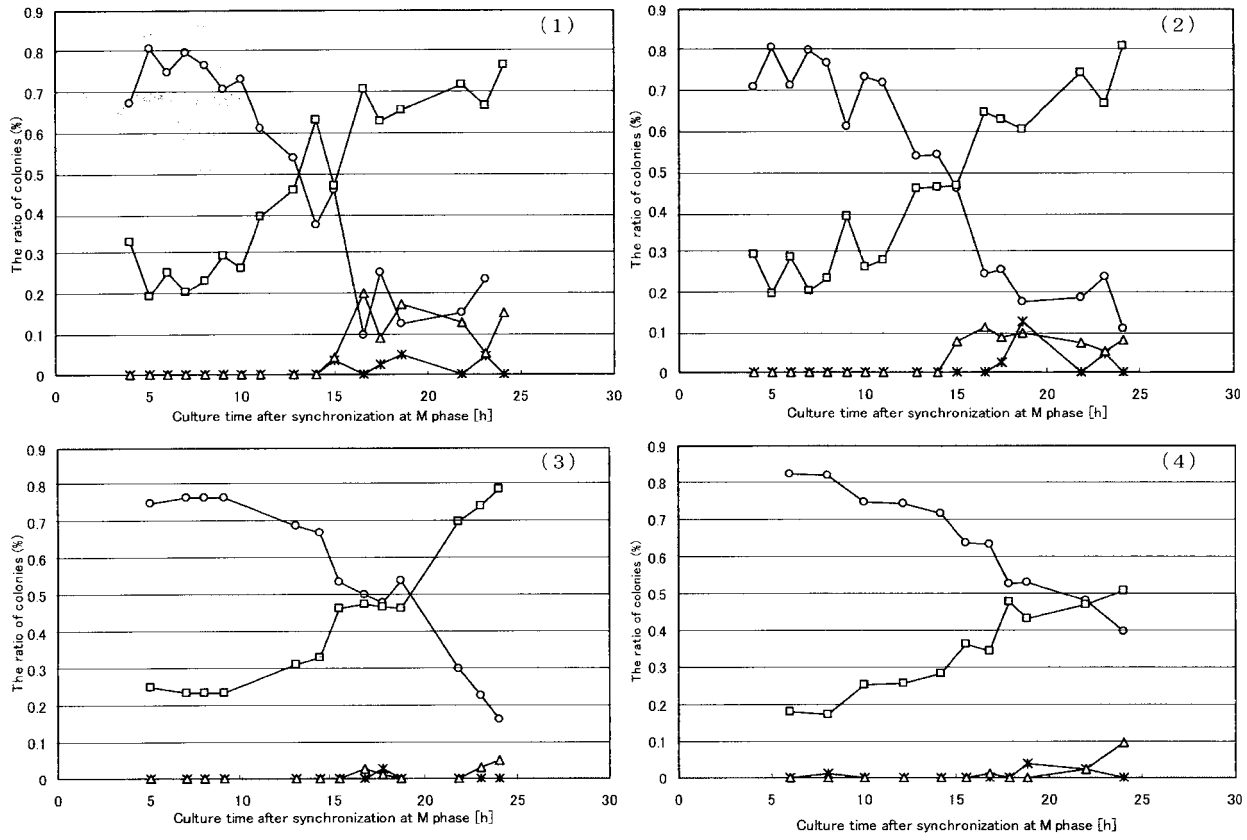

Fig. 2 Effect of the *C. nomame* extract on cell cycle.
Time dependence of variation of ratios of the colonies with different numbers of cells after cells were treated with or without MMC. Each mark shows the ratio of colonies with one (○), two (□), three (*) or four cells (△). (1) Cells were incubated in the basal medium without MMC pretreatment. (2) Cells were incubated in the presence of the extract without MMC pretreatment. (3) Cells were treated with MMC and then incubated in the basal medium. (4) Cells were treated with MMC and then incubated in the presence of the extract.

presence of the *C. nomame* extract. The ratio of two-cell colonies reached 50% at 18 h after the synchronization. Thereafter, increase of the ratio was not observed until 24 h after the synchronization. The first division of the cells incubated with the extract after MMC-treatment delayed more than without the extract.

Discussion

When CHO-K1 cells were treated with MMC (2.5μM) for 1 h, the frequency of metaphase cells with chromosome aberrations reached a maximum 15 h after MMC treatment (Fig.1). It is established that cells in G_1-phase are more susceptible to MMC than those in other phases. The doubling time of CHO-K1 cells used in the present experiments is known to be 11.5 h.[3] The result suggests that the MMC-treated G_1-cells take a longer time than non-treated G_1-cells to proceed through S- and G_2-phase into M-phase. It was reported that MMC prolonged cell cycle of CHO.[4] CHO cells described by Sasaki *et al.*[4] and in our previous report[2] have 17 h of the population doubling time when cultured in the basal medium, while they have 24 h when treated with MMC. When CHO-K1 cells were cultured in the presence of the *C. nomame* extract after MMC treatment, the frequencies of the metaphase cells with MMC-induced chromosome aberrations were significantly lowered (Fig.1). This result is in agreement with the previous report on CHO cells.[2] The suppressing effect of the extract on the chromosome aberrations was reconfirmed.

In the present experiment, it was shown that the peak of the frequency of chromosome aberrations induced by MMC appeared remarkably later in medium with the extract than in medium without the extract (Fig.1). The finding suggested that the extract not only suppressed chromosome aberrations induced by MMC, but also influenced the progress of cell cycle on the cells treated with MMC. Then we investigated the effect of the extract on the first division of cells by using synchronized cells at M-phase.

As shown in Fig.2-1 and 2-2, the extract seemed to have no effect on the normal cell cycle. Consequently, it is concluded that the extract itself has no effect on the first division cycle after the selection of M-phase cells.

MMC treatment was performed 2 h after the M-phase synchronization. It is plausible that the cells were treated with MMC in their G_1-phase, because the duration of M-phase and G_1-phase in CHO-K1 cells is 0.5 and 2.5 h, respectively.[3] As shown in Fig.2-3, the proportion of two-cell colonies did not increase during 5 h after the proportion reached 50 %. Thus, it is thought that a part of cells treated with MMC in G_1-phase were delayed in progress into M-phase. Comparison of the results of the experiments with (Fig.2-4) and without (Fig.2-3) the extract after MMC-treatment indicated that the extract further delayed the first division of the cells treated with MMC.

It was indicated that the extract can lower the frequency of the chromosome aberrations and influence the first division after MMC treatment. Based on the finding that the extract did not retard the division cycle unless cells were pretreated with MMC, it is suggested that the extract can not directly delay the division cycle. In the previous report,[2] we showed that the frequency of aberrant cells during incubation after MMC treatment reached the peak in the presence of the extract earlier than in the absence. These contrary findings suggest that the suppressing effect of the extract on chromosome aberrations induced by MMC have relation to the progression of cell cycle. It is possible that the efficacy of the extract on the progression of cells into the first M-phase is different depending on the stages in which cells are treated with MMC. More studies are needed to obtain a definite understanding on the mechanism of the extract in cell cycle and repair on chromosome aberration.

Acknowledgments

We would like to thank Associate Prof. Yû F. Sasaki, Department of Chemical and Biological Engineering, Hachinohe National College of Technology, Aomori (Japan), for his valuable advice in this study. We wish to thank J. Kaihara for her critical reading of the manuscript.

和文抄録

チャイニーズハムスター卵母K1株化細胞（CHO-K1）を，最終濃度2.5μMのマイトマイシンC（MMC）で1時間処理した後，ウシ胎児血清を10v/v%含むHam's F12培地を基本培地としてカワラケツメイ抽出物を添加あるいは添加しない条件で培養を行った。その結果，

MMCで染色体異常が生じる細胞の頻度は，カワラケツメイ抽出物を添加した方が有意に低かった。同調培養での実験の結果，カワラケツメイ抽出物は，MMCで処理したCHO-K1細胞について，処理後1回目の細胞分裂周期を遅らせることが分かった。カワラケツメイ抽出物がMMCで処理していない細胞の分裂周期に影響を与えないことから，カワラケツメイ抽出物がMMCで誘導された染色体異常を抑制する作用と細胞周期の遅延との間に何らかの関係があるものと考えられる。

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