ENHANCEMENT OF COLONY FORMATION OF MOUSE BONE MARROW CELLS BY UBENIMEX†

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Ubenimex enhanced colony formation of bone marrow cells from CDF_1 mice induced by L_{929} cell supernatant, which shows a macrophage-colony-stimulating activity (M-CSA), and also enhanced the colony formation induced CDF_1 mouse spleen cell conditioned medium, which shows a granulocyte and macrophage-colony-stimulating activity. The maximal effect was obtained at 0.01 μg/ml. But, ubenimex showed no effect on the nature of the colonies induced by each CSA. By preincubation of the bone marrow cells with ubenimex, M-CSA-induced colony-forming and the M-CSA-binding activities of the cells were increased. These results suggest that ubenimex enhances the CSA-induced colony formation of bone marrow progenitor cells of CDF_1 mouse by increasing the amount of the CSA-binding to the cells.

Ubenimex is an aminopeptidase inhibitor isolated from the culture filtrate of *Streptomyces olivoreticuli*[1]. It has been demonstrated to have antitumor[2~5] and immuno-modulating activities[2,6~10] in animals. Recently, ISHIZUKA *et al.* found an enhancing effect of ubenimex on colony formation of CDF_1 mouse bone marrow cells induced by a serum which was prepared from CDF_1 mice previously treated with lipopolysaccharide[6]. This serum contains a granulocyte-colony-stimulating activity (G-CSA)[11]. In the present paper, the effects of ubenimex on colony formation of bone marrow cells from CDF_1 mice induced by L_{929} cell supernatant containing a macrophage-colony-stimulating activity (M-CSA)[12] and by spleen cell conditioned medium containing a granulocyte and macrophage-colony-stimulating activity (GM-CSA)[13], are presented.

Materials and Methods

Female CDF_1 mice, 2 to 3 months old, were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan. Bone marrow cells were isolated from the femurs as described previously[6]. Ubenimex was supplied by Nippon Kayaku Co., Ltd.[14], dissolved in phosphate-buffered saline (pH 7.4) and sterilized by passing through a 0.22 μm Millipore membrane. The sterile stock solution (1 mg/ml) was diluted with the phosphate-buffered saline as required when used. [6-^{3}H]Thymidine (19.3 Ci/mmol) was obtained from New England Nuclear, Boston, U.S.A. M-CSA was prepared from L_{929} cells according to PELUS *et al.*[15] and GM-CSA from CDF_1 mouse spleen cells according to ONODA *et al.*[16]. Colony formation of bone marrow cells was carried out according to TSUNEOKA and SHIKITA[17] in 35-mm Falcon 3001 dishes. Bone marrow cells (1×10^5 cells/ml/dish) were incubated in McCOY's 5A medium containing 20% fetal calf serum (FCS) and 0.32% Bacto-agar. After the incubation at 37°C in 5% CO_2 in air for 7 days, colonies >50 cells per colony were counted microscopically. All data were analyzed by Student's t-test.

† Hereafter, by recommendation of WHO, the name of ubenimex is used for bestatin.

Results

The effects of ubenimex on M- and GM-CSA-induced colony formation of bone marrow cells were examined and the results are shown in Fig. 1. Ubenimex enhanced the M-CSA-induced colony formation significantly at concentrations of 0.001, 0.01 and 0.1 μg/ml and GM-CSA-induced colony formation at 0.001 and 0.01 μg/ml. The maximal enhancement was observed at 0.01 μg/ml in both cases. Ubenimex also enhanced uptake of [^{3}H]thymidine by bone marrow cells treated with M-CSA in parallel with the enhancement of the colony formation (Fig. 2). Ubenimex did not alter the relative number of macrophage and granulocyte in colonies by each stimulant (Table 1).

When the bone marrow cells were preincubated with ubenimex at 0.01 μg/ml, the CSA-induced colony formation was enhanced in all preincubation periods tested (8~40 hours) (Table 2). The enhancement of CSA-binding to cells was also observed in this experiment (Table 2). When bone marrow cells were preincubated for 16 hours with a high concentration of ubenimex (100 μg/ml), both colony-forming and CSA-binding activities were not enhanced (data not shown).

Fig. 1. Enhancement of colony formation of bone marrow cells induced by L_{929} cell supernatant or spleen cell conditioned medium by treatment of ubenimex.

Bone marrow cells were incubated in the presence of 100 units of L_{929} cell supernatant (M-CSA, ●) or 50 units of CDF_1 spleen cell conditioned medium (GM-CSA, ○). Each circle represents mean of triplicate data with SD.

* $P<0.05$ vs. control. ** $P<0.01$ vs. control.

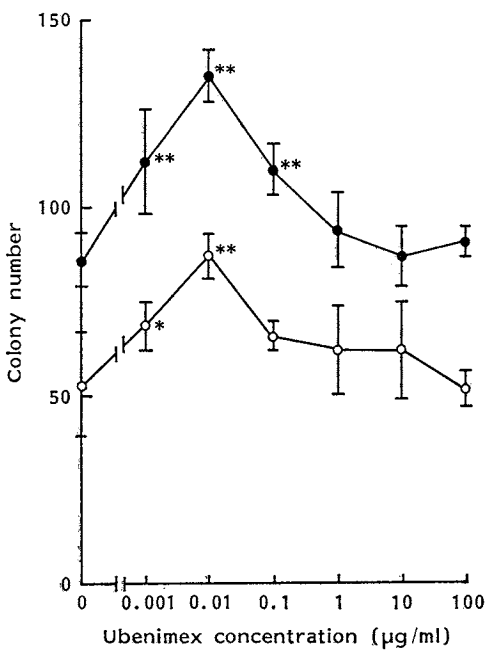

Fig. 2. Effect of ubenimex on the uptake of [^{3}H]-thymidine bone marrow cells in the presence of L_{929} cell supernatant.

The uptake was assayed according to PESSINA *et al.*[18]. Bone marrow cells (1×10^5 cells) were suspended in 0.2 ml of McCOY's 5A medium containing 20% FCS with 50 units of M-CSA and ubenimex at various concentrations. The incubation mixture was seeded in 96-well Costar plates and incubated at 37°C in 5% CO_2 in air for 72 hours. Twenty-four hours before completion of the incubation 1 μCi of [^{3}H]thymidine in 1 μl was added. Each circle represents mean of six replicate data with SD.

* $P<0.01$ vs. control.

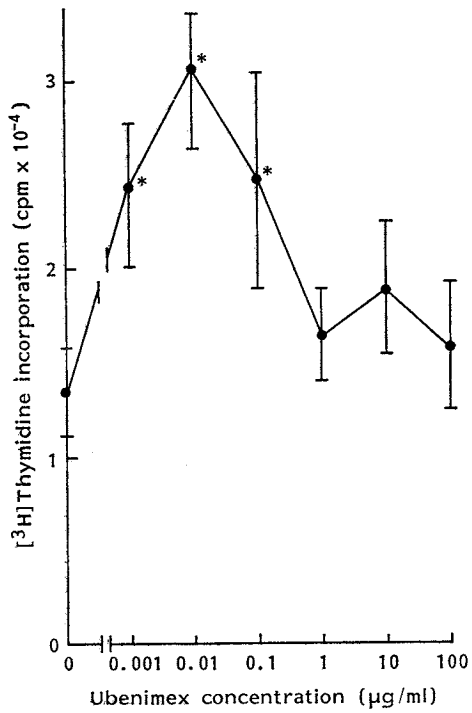

Table 1. The effect of ubenimex on the population of colonies formed by bone marrow progenitor cells in the presence of M-CSA or GM-CSA.

Source of CSA	Ubenimex (μg/ml)	Number of colony	Population of colony		
			Macrophage (%)	Granulocyte (%)	Both cells (%)
L_{929} cell	0	86±7	92±2	0	8±2
supernatant	0.01	135±7**	89±3	0	11±3
Spleen cell	0	65±11	63±4	24±4	13±1
supernatant	0.01	92±2*	64±3	23±2	13±2
—	0	0	0	0	0
	0.01	0	0	0	0

Colony formation was performed in the presence of 100 units of M-CSA or 50 units of GM-CSA. Morphologic examination of the colony-formed cells was done after the cells were stained by means of esterase activity[19]. Each value represents mean of triplicate data with SD.

* $P<0.05$. ** $P<0.01$.

Table 2. Effect of ubenimex on the colony-forming and CSA-binding activities of bone marrow cells.

Preincubation period (hours)	Colony-forming activity (number of colony)		CSA-binding activity (%)	
	Ubenimex		Ubenimex	
	0 μg/ml	0.01 μg/ml	0 μg/ml	0.01 μg/ml
8	58±5	78±7*	5.8	14.8
16	60±7	93±7*	16.1	30.3
24	73±5	142±7**	23.9	41.9
32	63±2	119±7**	23.9	43.2
40	51±7	94±4**	24.5	41.9

The conditions of preincubation was carried out according to CARRAMATTI *et al.*[20] in 6-ml Falcon 5045 tubes. Without any CSA, a quantity of 10^7 bone marrow cells was suspended in 1 ml of McCOY's 5A medium containing 20% FCS with or without ubenimex and preincubated at 37°C in 5% CO_2/air as indicated. Changes of the number and viability of the preincubated cells were not observed. One portion of the preincubated cells was subjected to the assay of colony-forming activity by measuring the colony formation in the presence of 100 units of M-CSA. These data are shown as mean±SD. Another portion of the preincubated cells was subjected to assay of M-CSA-binding activity. The preincubated cells (1×10^7 cells) were suspended in 1 ml of the above medium with 300 units of M-CSA and further incubated at 4°C for 2 hours in the 35-mm petri dishes. In the control run the cells were not added. After the incubation the supernate was separated by centrifuging at 1,200 rpm for 5 minutes, and the CSA remained in the supernate was assayed by colony formation of fresh bone marrow cells with 0.3 ml of the supernate. CSA-binding activity was calculated from the following equation.

$$\text{CSA-binding activity (\%)} = \left(1 - \frac{\text{Supernate CSA of sample}}{\text{Supernate CSA of control}}\right) \times 100$$

* $P<0.05$ vs. control. ** $P<0.01$ vs. control.

Discussion

Recently, ISHIZUKA *et al.* found an enhancing effect of ubenimex on colony formation of CDF_1 mouse bone marrow cells induced by G-CSA[8]. In the present study, L_{929} cell supernatant as a source of M-CSA and CDF_1 mouse spleen cell conditioned medium as a source of GM-CSA were used as the stimulant of bone marrow cells. We found that ubenimex enhanced colony formation of CDF_1 mouse bone marrow cells induced by M- and GM-CSA at a low concentration (0.01 μg/ml), although ubenimex by itself did not have any CSA. Ubenimex did not change the proportion of macrophages and granulocytes in the colonies induced by each stimulant.

When, in the absence of CSA, CDF_1 mouse bone marrow cells were preincubated with ubenimex

at the optimal concentration of 0.01 μg/ml, ubenimex enhanced the CSA-binding activity of the cells in addition to the CSA-induced colony-forming activity. From the results, it was speculated that this enhancement of the CSA-binding activity of the bone marrow progenitor cells by ubenimex closely related to the enhancement of the CSA-induced colony-forming activity. On the other hand, the similarly preincubated cells at the concentration of 100 μg/ml of ubenimex, which did not enhance each CSA-induced colony formation, did not show both activities significantly enhanced. These findings suggest that ubenimex enhances the CSA-induced colony formation of bone marrow progenitor cells by increasing the bound of CSA to the cells. The CSA-binding activities at 40 hours was as high as at 24 hours, but number of colonies decreased by 67% and 57% of that of 24 hours preincubation time with or without ubenimex. It is seemed that 40 hours of the preincubation period without CSA affects the ability of bone marrow cells to grow and differentiate.

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