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EFFECTS OF OKADAIC ACID ON MOUSE HEMOPOIETIC CELLS

Syuichi Oka[1*], Kazuya Nakagomi[1], Hideoki Tanaka[1], and Takeshi Yasumoto[2]

[1] Fermentation Research Institute, Agency of Industrial Science and Technology, Higashi 1-1-3, Tsukuba, Ibaraki 305, Japan

[2] Faculty of Agriculture, Tohoku University, Tsutsumi-dori, Sendai, Miyagi 980, Japan

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Effects of okadaic acid, a potent non-12-O-tetradecanoyl-phorbol-13-acetate(TPA)-type tumor promoter, on mouse hemopoietic cells were investigated. Okadaic acid stimulated mouse bone marrow cells to form granulocyte-macrophage colony-forming unit(CFU-GM) colonies without added colony stimulating factors(CSFs). At the concentration of 1.82×10^{-8}M, colony formation of 77 ± 14 colonies/1×10^5 bone marrow cells was observed. Observations on the effects of other cells on the CSF induction suggested that okadaic acid primarily stimulated the functions of macrophages, and the CSF production from macrophages might be attributed to the CFU-GM colony formation. On the other hand, the erythroid colony-forming unit(CFU-E) colony formation stimulated by erythropoietin was inhibited by the addition of okadaic acid.

Granulocytes, monocyte-macrophages, and erythrocytes are derived from the proliferation and differentiation of a common hemopoietic stem cell. Such a process of hemopoiesis requires colony stimulating factors(CSFs) and erythropoietin as proliferation and differentiation regulators, respectively.

In the investigations on bioactive substances of microbial origin that affect hematopoiesis, we found that the teleocidins stimulated mouse bone marrow cells to form granulocyte-macrophage colony-forming

* To whom correspondence should be addressed.

Abbreviations: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; CSF, colony stimulating factor; CFU-GM, granulocyte-macrophage colony-forming unit; CFU-E, erythroid colony-forming unit; IL-1, interleukin 1.

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unit(CFU-GM) colonies in the absence of CSF (1). Because the teleocidins bound to phorbol ester receptors in cell membranes and activated protein kinase C, these were classified as 12-O-tetradecanoyl-phorbol-13-acetate(TPA)-type tumor promoters (2).

Okadaic acid, though first isolated from sponges (3), was found to be a product of the benthic marine dinoflagellate Prorocentrum lima (4). Recently, okadaic acid was shown to be a potent inhibitor of phosphatases (5-10), and was further revealed to be a potent non-TPA-type tumor promoter (11). Unlike the teleocidins, okadaic acid did not bind to the phorbol ester receptors and did not activate protein kinase C. The tumor promoting activity of okadaic acid was comparable to that of TPA. Consequently, we investigated whether okadaic acid could stimulate CFU-GM colony formation and found that okadaic acid stimulated mouse bone marrow cells to form CFU-GM colonies without added CSFs, as do the teleocidins.

In this paper, we will describe the effects of okadaic acid on mouse hemopoietic cells and possible mechanisms for the CFU-GM colony formation.

MATERIALS AND METHODS

Animals and Materials: Male ICR mice, 6- to 10-weeks-old, were purchased from Charles River Japan, Inc. Okadaic acid was isolated from cultured cells of the dinoflagellate Prorocentrum lima as described previously (4). Horse serum was obtained from Gibco Laboratories. Fetal calf serum was obtained from HyClone Laboratories. CSF-CHUGAI, a partially purified preparation from a human squamous cell carcinoma cell line, was a product of Chugai Pharmaceutical Co., Ltd. Erythropoietin (from human urine, 72 U/mg) was purchased from Toyobo Co., Ltd. The other chemicals used were of analytical grade.

Assay for CFU-GM and CFU-E colony formation: CFU-GM and CFU-E colony formation was assayed as described in the previous paper (1). Methylcellulose cultures based on the modified methods of Iscove et al.(12) were carried out using bone marrow cells (1×10^5) obtained from ICR mice.

Preparation of peritoneal macrophages: Peritoneal exudate cells, obtained from male ICR mice 4 days after they had been intraperitoneally injected with 2 ml of 10% proteose peptone, were centrifuged at 140 x g for 5 min. The pellets were suspended in Hanks' balanced salt solution and washed twice with the same medium. The peritoneal cells were resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum and incubated in 16 mm wells at 1 x 10^6cells/well for 2 hrs at 37°C in 5% CO_2. The adherent macrophages were washed twice with the same medium and used for the culture experiments. Macrophages were cultivated in 1 ml of the medium with or without test samples at 37°C in a humidified atmosphere of 5% CO_2. The aliquots were centrifuged and assayed for CFU-GM colony formation.

Preparation of T cell-enriched fractions: The splenocytes obtained from male ICR mice (7- to 8-weeks-old) were suspended in Eagle's minimum essential medium and mashed on a stainless steel mesh (# 150). Then, a solution of 0.83% NH_4Cl / 0.17M Tris HCl buffer (pH 7.65) (9/1) was added and maintained at 4°C for 5 min to remove erythrocytes. After the centrifugation at 140 x g for 5 min, the cells were washed twice with Eagle's medium containing 5% fetal calf serum and passed through a Sephadex G-10 column . The cell fractions were centrifuged and then loaded on a column of nylon fiber equilibrated with Eagle's medium supplemented with 10% fetal calf serum. T cell-enriched fractions were collected and washed twice with RPMI 1640 medium supplemented with 5% fetal calf serum. The culture experiments were performed with 1 x 10^6 cells/1 ml of the same medium with or without test samples at 37°C for 3 days in a humidified atmosphere of 5% CO_2. The thus obtained cells reacted with anti-mouse Thy-1 and complement.

Preparation of B cell enriched fractions: The splenocytes that had been passed through a Sephadex G-10 column were treated with anti-mouse Thy-1 at 30°C for 30 min and followed by treatment with complement(1/5 dilution). Then, the cells were washed and used for the culture experiments. The thus obtained cells reacted with anti-mouse B cell.

RESULTS and DISCUSSIONS

The effects of okadaic acid on in vitro colony formation were examined. As shown in Fig. 1, okadaic acid stimulated CFU-GM colony formation in the concentration range between 1.82 x 10^{-8}M and 1.82 x 10^{-9}M in the absence of CSF. Okadaic acid at the concentration of 1.82 x 10^{-8}M stimulated the formation of 77 ± 14 colonies/1 x 10^5 bone marrow cells, while 5 units/ml of CSF-CHUGAI induced the formation of 27 ± 2 colonies/1 x 10^5 bone marrow cells. However, the addition of 1.82 x 10^{-7}M okadaic acid to the culture produced no colonies because of the cytotoxic effect of okadaic acid at this concentration. Although the

tumor promoting activity of okadaic acid was comparable to that of teleocidin (5), the ability of okadaic acid to stimulate CFU-GM colony formation was greater than h a t of teleocidin A-1. Okadaic acid at the concentration of 1.82×10^{-8}M resulted in the formation of 77 ± 14 colonies, while teleocidin A-1 at 5.00×10^{-8}M merely stimulated the formation of 56 ± 10 colonies.

Morphological analysis (non-specific and chloroacetate esterase dual staining) showed that the colonies induced by the addition of okadaic acid were composed of granulocytes and macrophages at 7 days of culture.

Because a number of mechanisms can be hypothesized to explain the effects of okadaic acid on myeloid colony formation in vitro, we decided to examine first the stimulatory effects on endogenous CSF production. As macrophages and lymphoid cells are the main producers of CSF, these cells were prepared and examined for the secretion of CSF.

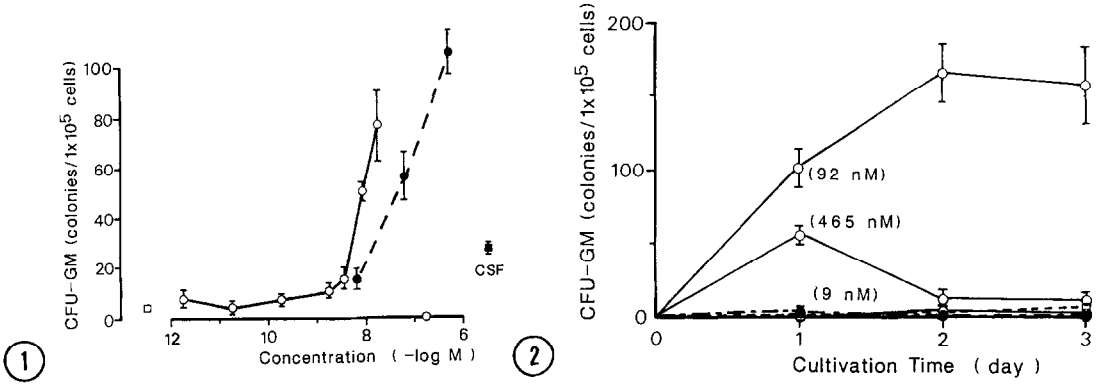

Fig. 1. Effect of okadaic acid on CFU-GM colony formation. Mouse bone marrow cells were cultured at $1x10^5$ cells/dish in the absence (□) or presence of CSF (■) at 5 U/ml, okadaic acid (O) or teleocidin A-1 (●) at various concentrations. Each circle represents the mean of triplicate data with S. E.

Fig. 2. Effect of okadaic acid on CSF production from peritoneal macrophages. Peritoneal macrophages ($1x10^6$ cells/well) were cultured with okadaic acid (O , 9 nM, 92 nM, 465 nM), teleocidin A-1 (● 250 nM) or RPMI 1640 medium (□) at 37°C in 5% CO_2 for 3 days. Fifty-microliter aliquots were assayed for CFU-GM colony formation. Each circle represents the mean of triplicate data with S. E.

Figure 2 shows the effect of okadaic acid on peritoneal macrophages induced by proteose peptone. The addition of okadaic acid to the macrophage cultures significantly stimulated CSF production. The maximal induction was obtained with the concentration of 9.2×10^{-8}M. The addition of higher doses reduced CSF production because of cytotoxicity. On the other hand, the addition of teleocidin A-1 to the macrophage cultures showed no stimulatory effect at the concentration of 2.5×10^{-7}M.

Next, T cell-enriched fractions were prepared from murine splenocytes and cultured with okadaic acid at 37°C in a humidified atmosphere of 5% CO_2 for 3 days. Although the addition of okadaic acid at the concentration of 9.20×10^{-8}M stimulated the formation of 14 ± 5 colonies/1×10^5 bone marrow cells, no significant production was observed.

Because B lymphocytes were reported to produce CSF (13,14), stimulatory effects of okadaic acid were examined. However, no stimulation was observed (data not shown).

Thus, a marked stimulation of myeloid colony formation was obserbed only by the addition of okadaic acid to the macrophage cultures. These observations suggest that okadaic acid primarily stimulates the functions of macrophages, and the CSF production from macrophages may be attributed to the CFU-GM colony formation. Recently, Hokama *et al.* showed that okadaic acid inhibited interleukin 1(IL-1) secretion from human peripheral blood monocytes at the concentrations used in the tumor promotion studies, while the addition of the lower concentrations of okadaic acid stimulated IL-1 secretion (15). Maximum stimulation was obtained at 0.05 μg/ml for 24- and 48-hr exposures. The concentrations that produced the maximum stimulation was almost the same as that

producing the maximal CSF secretion from mouse peritoneal macrophages. On the other hand, it has been reported that IL-1 induced CSF production from marrow accessory cells (16,17) and synergized with various CSFs to stimulate myeloid colony formation (18). Therefore, a possible mechanism for the CFU-GM colony formation by the addition of okadaic acid to the bone marrow culture could be deduced as follows: Okadaic acid induces CSF and IL-1 production from marrow cells and then, these cytokines synergistically enhance CFU-GM colony formation. With respect to the participation of phosphatase activities, further investigations are in progress.

Then, the effects of okadaic acid on erythroid progenitors were examined. The addition of okadaic acid to the methylcellulose culture showed no stimulatory effect on erythroid colony formation in the absence of erythropoietin in vitro (data not shown). However, okadaic acid inhibited erythroid colony-forming unit(CFU-E) colony formation stimulated by 0.1 U/ml of erythropoietin in vitro as shown in Fig. 3. In the concentration range between 3.60×10^{-9}M and 1.82×10^{-8}M, the CFU-E colony formation was significantly inhibited, whereas CFU-GM

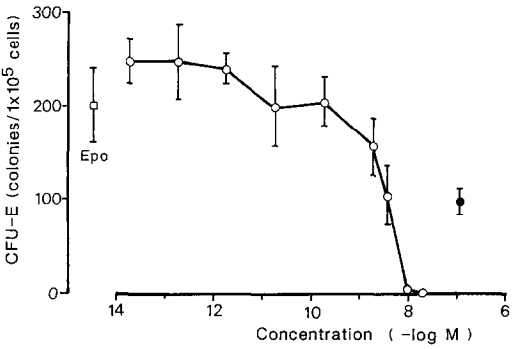

Fig. 3. Effect of okadaic acid on CFU-E colony formation. Mouse bone marrow cells ($1x10^5$ cells/dish) were cultured with 0.1 U/ml erythropoietin (Epo) in the absence (□) or presence of okadaic acid (O) or teleocidin A-1 (●) at various concentrations. Each circle represents the mean of triplicate data with S. E.

colony formation was markedly stimulated in this concentration range. Although teleocidin A-1 added to the methylcellulose cultures also inhibited erythropoietin-induced CFU-E colony formation (1), it was less potent than okadaic acid.

Broudy et al. reported that TPA caused a decrease in the number of erythropoietin receptors without altering the affinity of the receptor for erythropoietin (19). Because the action of okadaic acid is presumably mediated by a receptor distinct from the TPA receptor (10), it is interesting to elucidate its mode of action on erythroid progenitors. Further experiments are under way.

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