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Induction of differentiation of human leukemia cells by inhibitors of myosin light chain kinase

Makoto Makishima[1], Yoshio Honma[1], Motoo Hozumi[1], Kazumi Sampi[1], Masao Hattori[1] and Kazuo Motoyoshi[2]

[1]*Saitama Cancer Center, Ina-machi, Saitama-362* and [2]*National Defense Medical College, Tokorozawa, Saitama-359, Japan*

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Inhibitors of myosin light chain kinase, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride (ML-9) and 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride (ML-7), induced Nitroblue tetrazolium reducing activity, lysozyme activity and morphological maturation of human monoblastic U937, THP-1 and promyelocytic HL-60 cells, but not of erythroblastic K562 cells. However, three analogs of ML-9, which are an inhibitor and an activator of protein kinase C, and a calmodulin antagonist, respectively, did not induce differentiation of the cells.

Differentiation; Myosin light chain kinase; ML-9; ML-7; Leukemia cell; U937 cell; HL-60 cell

1. INTRODUCTION

Protein kinase activities are important in the regulation of biological activities of cells such as proliferation and differentiation [1,2]. Extracellular signals are translated into intracellular messenger systems through tyrosine protein kinase, phosphatidylinositol turnover and GTP binding protein [3]. Activation of phosphatidylinositol turnover increases the level of diacylglycerol, which is an activator of protein kinase C (PKC), and of inositol triphosphates, which increase the intracellular Ca^{2+} level [3,4]. This Ca^{2+} then binds to calmodulin and the Ca^{2+}–calmodulin complex activates further enzymes such as myosin light chain kinase (MLCK) [5,6]. PKC and MLCK are serine/threonine protein kinases [2].

Inhibitors of these signal transduction pathways are very useful in studies on the mechanisms of cellular growth and differentiation. Herbimycin A, an inhibitor of protein tyrosine kinase, induced differentiation of human erythroleukemic K562 cells, which have high tyrosine kinase activity of $p210^{bcr\text{-}abl}$ protein [7], and methyl 2,5-dihydroxycinamate, another inhibitor of protein tyrosine kinase, enhanced the differentiation of human myeloblastic ML-1 cells induced by vitamin D_3 [8]. An inhibitor of phosphatidylinositol turnover, psi-tectorigenin, and phorbol esters, which are activators of PKC, also induced the differentiation of human leukemia cells [8,9]. Therefore, in this study, we examined the effects of various inhibitors of protein kinases on growth and differentiation of human myeloid leukemia cell lines, and found that inhibitors of MLCK induced functional and morphological differentiation of some cell lines.

Abbreviations: PKC, protein kinase C; MLCK, myosin light chain kinase; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride; ML-7, 1-(5-iodonapthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; SC-9, *N*-(6-phenylhexyl)-5-chloro-1-naphtalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; NBT, Nitroblue tetrazolium

Correspondence address: Makoto Makishima, Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Saitama-362, Japan. Fax: (81) (48) 722 1739.

2. MATERIALS AND METHODS

2.1. *Materials*

1-(5-Chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride (ML-9), 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride (ML-7), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) were purchased from Seikagaku Kogyo, Tokyo. Stock solutions were prepared in dimethyl sulfoxide and diluted with ethanol before use. The final concentration of dimethyl sulfoxide did not exceed 0.1% (v/v).

2.2. *Cell lines and cell culture*

Human leukemia U937 [10], HL-60 [11], THP-1 [12], K562 [13] and HEL [14] cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% Co_2 in air.

2.3. *Assay of cell growth and properties of differentiated cells*

Suspensions of cells (10^5 cells/ml) in 2 ml of culture medium were cultured with or without test compounds in multidishes (Costar, Cambridge, MA) for 5 days. Then cell numbers were counted in a Model ZM Coulter Counter (Coulter Electronics, Luton, UK). Nitroblue tetrazolium (NBT) reduction was assayed colorimetrically as reported previously [15]. Washed cells were sonicated for 10 s in a cell disruptor (Ultrasonics, NY) and their cytosolic lysozyme activities were determined by a lysoplate method [16]. Erythroid differentiation

was scored by benzidine staining [17]. Cell morphology was examined in cell smears stained with May-Gruenwald-Giemsa stain. Naphthol AS-D chloroacetate esterase and α-naphthol acetate esterase were determined cytochemically with an esterase kit (Sigma Chemical Co., St. Louis).

3. RESULTS

Human monoblastic leukemia U937 cells were cultured with various concentrations of ML-9, a potent inhibitor of MLCK [18,19]. ML-9 inhibited proliferation of the cells, its concentration for 50% inhibition of growth (IC_{50}) being 21 μM, and induced NBT reducing activity concentration-dependently (Fig. 1a). ML-7, another inhibitor of MLCK [19], also induced NBT reducing activity. H-7 and SC-9 are an inhibitor and an activator of PKC, respectively, and have structures similar to ML-9 and ML-7 [20,21]. However, neither H-7 nor SC-9 induced NBT reducing activity of the cells. Although W-7 is a calmodulin inhibitor with structural similarity to ML-9 and inhibits MLCK indirectly [22], it did not induce NBT reducing activity of U937 cells. The IC_{50} concentrations of ML-7, H-7, SC-9 and W-7 were 25 μM, 30 μM, 20 μM and 15 μM, respectively (data not shown). The lysozyme activity of U937 cells was also increased by treatment with ML-9 and ML-7, but not H-7, SC-9 or W-7 (Fig. 1b). On treatment with ML-9 or ML-7 for 7 days, monoblastic U937 cells differentiated into monocytic cells with increased activities of both naphthol AS-D chloroacetate esterase and α-naphthol acetate esterase (data not shown).

Next, we investigated the effect of ML-9 on the differentiation of other human leukemia cell lines. THP-1 and HL-60 cells are monoblastic and promyelocytic cells, respectively [11,12], and have been reported to differentiate on treatment with various inducers of differentiation [9]. ML-9 induced NBT reducing and lysozyme activities in THP-1 cells as well as U937 cells (Table I). ML-9 enhanced the lysozyme activity of HL-60 cells only slightly, but it induced morphological differentiation of the cells into metamyelocyte-like cells (data not shown). HEL and K562 cells are erythroblastic cells and differentiate into erythrocytic or myelomonocytic cells on treatment with inducers [9,23]. ML-9 did not augment hemoglobin synthesis in HEL or K562 cells (data not shown), but induced NBT reducing activity in HEL cells (Table I).

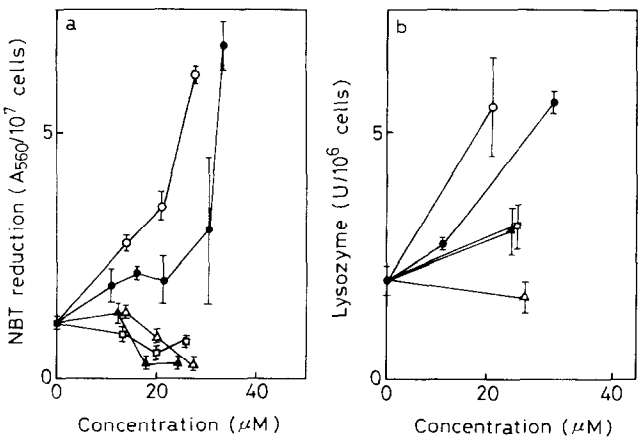

Fig. 1. Effects of inhibitors of MLCK and related compounds on induction of NBT reducing activity (a) and lysozyme activity (b) of U937 cells. The cells were treated with various concentrations of ML-9 (○), ML-7 (●), H-7 (△), SC-9 (▲) or W-7 (□) for 5 days. Values are means ± SD for three separate experiments.

Table I

Effects of ML-9 on growth and differentiation of several human leukemia cell lines

Cells	Growth inhibition IC_{50}[a] (μM)	NBT reduction[b] (ratio)	Lysozyme activity[b] (ratio)
U937	21	3.0	2.8
THP-1	29	7.5	4.2
HL-60	8	1.3	1.5
K562	8	1.1	ND[c]
HEL	14	2.0	ND

[a]IC_{50}, concentration of ML-9 required for 50% inhibition of cell growth in 5 days.

[b]Cells were cultured with the IC_{50} concentration of ML-9 for 5 days. The NBT reducing activities of untreated U937, THP-1, HL-60, K562 and HEL cells were 1.16 ± 0.14, 0.48 ± 0.06, 0.78 ± 0.04, 1.35 ± 0.15 and 1.04 ± 0.09 $A_{560}/10^7$ cells, respectively. The lysozyme activities of untreated U937, THP-1 and HL-60 cells were 2.0 ± 0.3, 6.1 ± 0.5 and 33.0 ± 10.2 U/10^6 cells, respectively.

[c]ND, not determined.

4. DISCUSSION

ML-9 has been reported to inhibit MLCK selectively with a K_i value of 3.8 μM and to affect the biological functions of platelets and smooth muscle cells [18,19]. However, there is no report on the relation of MLCK activity with cellular differentiation. Therefore, we examined the effect of ML-9 on differentiation of human leukemia cells. ML-9 induced differentiation of U937 and other leukemia cells, and another MLCK inhibitor, ML-7, also did. PKC is reported to phosphorylate myosin light chain by a different mechanism from that of MLCK [24]. Therefore, we examined the effect of H-7, which is structurally related to ML-9 and inhibits PKC [20]. However, H-7 did not induce differentiation of the U937 cells, although it induced differentation of mouse megakaryoblastic C1 cells [25]. SC-9, which is also structurally related to ML-9, is an activator of PKC [21]. SC-9 did not induce cell differentiation, whereas phorbol esters, which are also activators of PKC, are known to be potent inducers of differentiation of human leukemia cells such as HL-60 and U937 [9]. The Ca^{2+}-calmodulin complex activates MLCK and W-7, a calmodulin antagonist, inhibits MLCK indirectly [22], but it did not induce differentiation of U937 cells. This may be due to a difference in types of MLCKs,

calmodulin-dependent and independent types [6], although other possibilities cannot be ruled out.

U937 cells were reported to differentiate by genistein, an inhibitor of protein tyrosine kinase and phosphatidylinositol turnover, and psi-tectorigenin [8], but not by herbimycin A [7], methyl 2,5-dihydroxycinamate [8], staurosporin, sphingosine or A-3 (data not shown). Staurosporin and sphingosine inhibit PKC as well as H-7 [26,27] and A-3 inhibits cyclic nucleoside-dependent protein kinase, one of the serine/threonine kinases [28]. In this study we showed that inhibitors of MLCK induced the differentiation of U937 cells, and these results suggest that differentiation of human leukemia cells is associated with inhibition of MLCK as well as inhibition of tyrosine protein kinase and phosphatidylinositol turnover. Further studies are in progress to elucidate the association of the tyrosine and serine/threonine kinase activities with cellular differentiation more precisely.

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