A Role for Calmodulin in the Growth of Human Hematopoietic Progenitor Cells

By Naoyuki Katayama, Masakatsu Nishikawa, Fumihiko Kornada, Nobuyuki Minami, and Shigeru Shirakawa

A possible role for calmodulin in the colony growth of human hematopoietic progenitor cells was investigated using pharmacologic approaches. We obtained evidence for a dose-dependent inhibition of colony formation of myeloid progenitor cells (CFU-C) stimulated by interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), or granulocyte CSF (G-CSF) by three calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), N-(4-aminobuty1)-5-chloro-2-naphthalenesulfonamide hydrochloride (W-13), and trifluoperazine. Chlorine-deficient analogs of W-7 and W-13, with a lower affinity for calmodulin, did not inhibit the growth of CFU-C colonies. W-7, W-13, and trifluoperazine inhibited the colony formation of immature erythroid progenitor cells (BFU-E) stimulated by IL-3 plus erythropoietin (Ep) or GM-CSF plus Ep, in a dose-dependent manner, while they did not affect the colony formation of mature erythroid progenitor cells (CFU-E) induced by Ep. W-7, W-13, and trifluoperazine also led to a dose-dependent inhibition of GM-CSF-induced colony formation of KG-1 cells. Calmodulin-dependent kinase activity derived from the KG-1 cells was inhibited by these three calmodulin antagonists in a dose-dependent manner. These data suggest that calmodulin may play an important regulatory role via a common process in the growth of hematopoietic progenitor cells stimulated by GM-CSF, and G-CSF.

Mechanisms related to the growth signal of Ep apparently are not associated with calmodulin-mediated systems.

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concentrations. Trifluoperazine (Sigma Chemical Company, St Louis, MO) was reported to be an antagonist for calmodulin-stimulated reactions.\textsuperscript{26} This compound was initially dissolved in ethanol and diluted in culture medium.

\textit{Semisolid culture.} CFU-C assay was performed using a modification of the technique originally described by Pike and Robinson.\textsuperscript{27} Bone marrow cells were suspended at $2 \times 10^8$/mL in Iscove's modified Dulbecco's medium (IMDM) (GIBCO) containing 0.3% agar (Difco Laboratory, Detroit, MI), 20% FCS (GIBCO), and CSFs at optimal concentrations (100 U/mL rhIL-3, 10 ng/mL rhGM-CSF, or 10 ng/mL rhG-CSF). After incubation for 14 days at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} in air, colonies containing more than 40 cells were counted using an inverted microscope. The number of colonies represents the mean of triplicate cultures. In some experiments, the cultures were transferred onto slide glass and stained using the method of Kubota et al.,\textsuperscript{28} with some modification. CFU-E colonies were grown in 1 mL IMDM containing $2 \times 10^8$ bone marrow cells, 0.8% methylcellulose (Nakarai Chemicals, Kyoto, Japan), 20% FCS, 1% bovine serum albumin (BSA) (Sigma), and 1 U/mL rh erythropoietin (rhEp), as described by Ogawa et al.,\textsuperscript{29} with modification. After incubation for 7 days, the number of colonies (aggregates of more than eight benzidine-positive cells) was scored. Colony counts represent the mean of duplicate cultures. The assay of BFU-E was performed at a cell concentration of $1 \times 10^7$/mL. Cells were cultured in IMDM containing 0.8% methylcellulose, 30% FCS, 1% bovine serum albumin (BSA), 2 U/mL rhEp, and an optimal concentration of IL-3 (100 U/mL) or rhGM-CSF (10 ng/mL). Colonies containing more than 100 cells on days 10 through 12 and on days 18 through 20 were scored as late BFU-E and early BFU-E, respectively. The assay was performed in duplicate cultures.

\textit{Clonal assay for a human myeloid leukemic cell line.} For the clonal assay, KG-1 cells were cultured in 96-well microtest culture plates with a flat bottom (Costar, Cambridge, MA) at concentrations of $10^4$/mL in IMDM containing 0.8% methylcellulose, 20% FCS, and 10 ng/mL of rhGM-CSF, as described.\textsuperscript{30} On day 7, colonies consisting of more than 40 cells were counted.

\textit{Fractionation of protein kinases.} KG-1 cells were washed extensively with serum-free RPMI 1640, then suspended in ice-cold 20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L DTT, 2 mmol/L EDTA, 0.25 mol/L sucrose, 2 mmol/L Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, 0.4 mmol/L phenylmethylsulfonyl fluoride (PMSF) (75 U/mL), leupeptin (10 mmol/L), and pepstatin A (5 mmol/L). The cells were homogenized with a glass-glass Potter-Elvehjem homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 1,000 rpm for 5 minutes at 4°C, and the supernatant (cytosol fraction) was centrifuged at 100,000 × g for 60 minutes. This preparation was applied to a DEAE-cellulose column equilibrated with sample buffer minus sucrose; the enzymes were eluted with a linear gradient of NaCl (0 to 350 mmol/L). Protein kinase C and calmodulin-dependent kinase were eluted at 50 to 100 and 200 to 250 mmol/L NaCl, respectively, on DEAE-cellulose column chromatography (Fig 1). The fractions containing protein kinase C or calmodulin-dependent protein kinase activity were pooled and concentrated with an Amicon concentration system (Amicon Corp, Lexington, MA).\textsuperscript{32}

\textit{Assay of protein kinases.} Protein kinase C activity was examined using histone H1 (Boehringer Mannheim, Indianapolis, IN), as described.\textsuperscript{33} Calmodulin-dependent kinase activity was examined using casein (Sigma) as a substrate, according to the method of Schuetz et al., with modification.\textsuperscript{34} The reaction mixture contained 20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L MgCl\textsubscript{2}, 0.5 mmol/L CaCl\textsubscript{2}, 50 mmol/L (γ-P\textsubscript{32}P)\textsubscript{ATP}, 3 mmol/mL casein, and appropriate amounts of enzyme fraction, with or without 1 mmol/L calmodulin. The reaction was terminated by adding 20% trichloroacetic acid. The acid-precipitable material was collected on Millipore filters (Millipore Corp, Bedford, MA) and counted in a liquid scintillation spectrometer.

\textit{Statistical analysis.} Student's t test was used to determine the statistical significance.

\section*{RESULTS}

\textit{Effects of naphthalenesulfonamide derivatives and trifluoperazine on CFU-C colony formation.} In attempts to elucidate whether or not calmodulin plays a role in CFU-C colony formation, naphthalenesulfonamide derivatives, W-7 and W-5, were added to the cultures. The calmodulin antagonist, W-7, inhibited the ability of CFU-C to form colonies with rhIL-3, rhGM-CSF, or rhG-CSF, in a dose-dependent manner, at concentrations from 1 to 20 μmol/L. The inhibitory patterns were comparable among rhIL-3, rhGM-CSF, and rhG-CSF. In contrast, W-5, a chlorine-deficient analogue of W-7 with a low affinity for calmodulin, failed to inhibit the growth of CFU-C colonies at the same ranges of concentrations of W-7 (Fig 2A). W-13, another naphthalenesulfonamide, is a potent antagonist for calmodulin, while the chlorine-deficient analog of W-13, W-12, is inactive for calmodulin. The addition of W-13 to cultures induced a dose-dependent inhibition of the growth of CFU-C colonies stimulated by rhIL-3, rhGM-CSF, or rhG-CSF, whereas no significant inhibition was observed in cultures to...
Fig 2. Effects of calmodulin antagonists W-7 and W-5 (A); W-13 and W-12 (B); and trifluoperazine (C) on CFU-C growth induced by rhIL-3, rhGM-CSF, or rhG-CSF. Results are given as the mean percentage (±1 SE) of the number of CFU-C in each control culture. Data in (A) were obtained from the same five separate experiments as those in (C). The numbers of CFU-C induced by rhIL-3, rhGM-CSF, or rhG-CSF per 2 × 10^5 nonadherent bone marrow mononuclear cells were 59 ± 13, 127 ± 26, and 118 ± 31, respectively. Data in (B) were obtained from the other five separate experiments. The numbers of CFU-C induced by rhIL-3, rhGM-CSF, or rhG-CSF per 2 × 10^5 nonadherent bone marrow mononuclear cells were 64 ± 12, 102 ± 20, and 96 ± 9, respectively. The IC_{50} of W-7 was 10 (rhIL-3), 10 (rhGM-CSF), and 10 (rhG-CSF) pmol/L. The IC_{50} of W-13 was 7 (rhIL-3), 6 (rhGM-CSF), and 6 (rhG-CSF) pmol/L. The IC_{50} of trifluoperazine was 8 (rhIL-3), 11 (rhGM-CSF), and 9 (rhG-CSF) pmol/L. Equal volumes of ethanol in dissolving 10 and 20 pmol/L trifluoperazine (○) did not affect the growth of CFU-C.

which W-12 had been added (Fig 2B). W-13 inhibited the growth of CFU-C colonies induced by rhIL-3, rhGM-CSF, and rhG-CSF to a similar extent. These results obtained with W-13 and W-12 were consistent with those obtained with W-7 and W-5.

To research further the possible involvement of calmodulin in the growth of CFU-C colonies, we used trifluoperazine as an alternative antagonist for calmodulin. When trifluoperazine was incorporated into the cultures, there was a dose-dependent inhibition of CFU-C colony formation (Fig 2C), to an extent similar to that seen with W-7 or W-13. As shown in Fig 2C, the medium containing an equal volume of ethanol used in dissolving 10 and 20 μmol/L trifluoperazine had no apparent effect on the number of CFU-C colonies, thereby suggesting that the inhibitory effects of trifluoperazine were not due to the ethanol present in the culture medium. The inhibitory patterns were comparable among rhIL-3, rhGM-CSF, and rhG-CSF.

Effects of W-7, W-13, and trifluoperazine on morphology of colonies stimulated by rhIL-3 or rhGM-CSF. To determine whether the inhibitory effects of calmodulin antagonists on the growth of CFU-C colonies are specific for a certain cell lineage, morphologic assessment of colony types was performed at concentrations that led to ~50% inhibition of the number of CFU-C colonies. We used rhIL-3 and rhGM-CSF since these two CSFs induce neutrophil (G), neutrophil and macrophage (GM), macrophage (M), and eosinophil (Eos) colonies. As shown in Tables 1 and 2,
Table 1. Effect of W-7 and Trifluoperazine on Morphology of Colonies Induced by rhIL-3 or rhGM-CSF

<table>
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<th>CSF</th>
<th>Drug Concentration (μmol/L)</th>
<th>Colony No.*</th>
<th>Colony Type (%)</th>
<th>Drug Concentration (μmol/L)</th>
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<th>Colony Type (%)</th>
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Abbreviations: G, neutrophils; M, macrophages; GM, neutrophils and macrophages; Eos, eosinophils.

*Mean number from triplicate cultures.

morphologic examination of the colonies induced by rhIL-3 or rhGM-CSF in the presence of calmodulin antagonists, W-7, W-13, or trifluoperazine, showed no significant differences in the extent of inhibition of particular cell lineages.

Effects of calmodulin antagonists on CFU-E colony formation. To determine whether or not calmodulin antagonists affect the growth of CFU-E colonies, bone marrow cells were cultured with rhEp in the presence of calmodulin antagonists. In contrast to the results obtained with CFU-C, W-7, W-13, and trifluoperazine had no apparent effects on CFU-E colony formation when used at concentrations shown to be inhibitory for the growth of CFU-C colonies induced by rhIL-3, rhGM-CSF, or rhG-CSF (Fig 3).

Effects of calmodulin antagonists on BFU-E colony formation. To study the effects of calmodulin antagonists on late and early BFU-E colony formation, rhIL-3 or rhGM-CSF was added to cultures containing rhEp (2 U/mL). As shown in Fig 4, a dose-dependent inhibition of late and early BFU-E colony formation was seen for calmodulin antagonists, W-7, W-13, and trifluoperazine. W-5 or W-12 exerted no effects on BFU-E colony formation. The degree of inhibitory effects by calmodulin antagonists did not differ between the cultures containing rhIL-3 plus rhEp, or rhGM-CSF plus rhEp. Interestingly, in a comparison with late BFU-E, colony formation of early BFU-E, which are generally regarded to be more immature erythroid progenitors, was inhibited to a significantly greater extent by calmodulin antagonists.

Effects of day 7 addition of calmodulin antagonists on CFU-C colony formation induced by rhG-CSF. Table 3

Table 2. Effects of W-13 on Morphology of Colonies Induced by rhIL-3 or rhGM-CSF

<table>
<thead>
<tr>
<th>CSF</th>
<th>Concentration of W-13 (μmol/L)</th>
<th>Colony No.*</th>
<th>Colony Type (%)</th>
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<th>Colony Type (%)</th>
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Abbreviations: G, neutrophils; M, macrophages; GM, neutrophils and macrophages; Eos, eosinophils.

*Mean number from triplicate cultures.
Effects of calmodulin antagonists on colony size of CFU-C induced by rhG-CSF, and BFU-E induced by rhIL-3 and rhEp. To characterize the effects of calmodulin antagonists on colony size, we enumerated the frequency of large colonies (Table 4). We used rhG-CSF as a source of CSF. The frequency of large CFU-C (greater than 100 cells) and large BFU-E (greater than 500 cells) in response to rhG-CSF and rhIL-3 plus rhEp, respectively, was decreased in the presence of calmodulin antagonists in comparison with that observed in their absence. Thus, these findings demonstrate that calmodulin antagonists resulted in a reduction not only in colony number but also in colony size.

Effects of W-7, W-13, and trifluoperazine on clonal growth of KG-1 cells stimulated by rhGM-CSF, and on Ca²⁺/calmodulin-dependent protein kinase and protein kinase C activities from KG-1 cells. To determine whether the inhibitory effects of calmodulin antagonists were due to a direct effect on hematopoietic progenitor cells, the effects of W-7, W-13, or trifluoperazine were examined using the human myeloid leukemia cell line, KG-1, as homogenous target cells. This cell line was found to proliferate in the presence of rhGM-CSF. The addition of W-7, W-13, and trifluoperazine to cultures inhibited the clonal growth of KG-1 cells with rhGM-CSF (Fig 5).

We then examined the effects of these three calmodulin antagonists on calmodulin-dependent kinase activity from KG-1 cells. The dose-dependent inhibition of calmodulin-dependent kinase activity by all three calmodulin antagonists was evident, as determined by in vitro phosphorylation of casein (Fig 6A). These inhibitory effects were comparable with those observed in the clonal assay.

To test the specificity of calmodulin antagonists, the effects of calmodulin antagonists of protein kinase C activity from KG-1 cells were assessed. As shown in Fig 6B, protein kinase C activity was unaffected by W-7 or W-13 at concentrations seen to be inhibitory for calmodulin-dependent protein kinase activity, while trifluoperazine inhibited protein kinase C activity to a lesser extent than calmodulin-dependent protein kinase activity. The ability of trifluoperazine to inhibit the colony formation correlated well with the potency in inhibiting calmodulin-dependent protein kinase activity rather than that in inhibiting protein kinase C activity, thereby suggesting that the predominant action exerted by trifluoperazine is probably mediated by interfering with calmodulin but not with protein kinase C. These findings provide support for the specificity of the calmodulin antagonists used in this study.

DISCUSSION

While cloning of genes encoding hematopoietic growth factors and the subsequent expression of their recombinant protein provided information on biologic activities, very little is known of the molecular mechanisms involved in hematopoiesis. The fundamental question concerns which molecule is an intracellular signal. In this study, we examined the role of calmodulin in the growth of human hematopoietic progenitor cells, since this compound is a Ca²⁺-receptive protein and is implicated in a variety of cellular processes.11-13,34-36
Calmodulin antagonists, W-7 and W-13, led to an inhibition of the growth of CFU-C colonies induced by IL-3, GM-CSF, or G-CSF in a dose-dependent manner, while their weaker analogs, W-5 and W-12, did not (Fig 2). These results also indicate that the affinity of the compounds to bind to calmodulin is generally related to their efficacy in inhibiting the growth of CFU-C colonies. Another calmodulin antagonist, trifluoperazine, gave comparable results (Fig 2). The calmodulin antagonists used did not affect CFU-E colony formation stimulated by Ep (Fig 3), and the delayed addition was found to be inhibitory for the formation of CFU-C colony with G-CSF (Table 3); hence, the inhibitory effects of these calmodulin antagonists on CFU-C are not toxic. Thus, calmodulin is probably an important regulatory

![Graphs showing effects of calmodulin antagonists on BFU-E growth](https://example.com/fig4.png)

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**Table 3. Effects of Delayed (day 7) Addition of Calmodulin Inhibitors on CFU-C Formation Induced by rhG-CSF**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>W-7 (10 μmol/L)</th>
<th>W-13 (10 μmol/L)</th>
<th>Trifluoperazine (10 μmol/L)</th>
<th>W-7 (10 μmol/L)</th>
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*Mean ± SD from triplicate cultures.*
component for the mechanism related to the growth of CFU-C stimulated by IL-3, GM-CSF, or G-CSF. The inhibitory effects of calmodulin antagonists on the growth of CFU-C colonies did not differ among distinct hematopoietic growth factors, IL-3, GM-CSF, and G-CSF. This observation raises the possibility that calmodulin-mediated systems may be commonly related to the process of growth of CFU-C, induced by IL-3, GM-CSF, or G-CSF. In addition, inhibition in the number of IL-3– or GM-CSF–induced colonies by the calmodulin antagonists was not associated with any change in particular colony types (Tables 1 and 2), thereby indicating that inhibition of the growth of CFU-C colonies by calmodulin antagonists does not appear to be due to a lineage-specific event in the myeloid pathway. Thus, calmodulin seems to play a common regulatory role in mechanisms linked to the growth of myeloid progenitor cells.

We observed no significant effect of calmodulin antagonists on the formation of CFU-E colony with Ep (Fig 3); hence, the calmodulin-mediated systems are probably not involved in the process of signal transduction of Ep. On the other hand, the calmodulin antagonists inhibited BFU-E colony formation in a dose-dependent manner in the presence of IL-3 plus Ep or GM-CSF plus Ep (Fig 4). Thus, the calmodulin-mediated system does not appear to be specific for the myeloid pathway but may be involved in the growth of immature progenitor cells during erythroid differentiation processes. Furthermore, the calmodulin antagonists exerted a different effect on late and early BFU-E, being more strongly inhibitory for the growth of early BFU-E. The growth of immature erythroid progenitor cells is generally regarded as being more dependent on IL-3 or GM-CSF. There is general agreement that mature erythroid progenitor cells are sensitive and respond to Ep but not to IL-3 or GM-CSF. Thus, the results raise the possibility that the calmodulin-dependent pathway serves important roles in signal transduction systems of IL-3, GM-CSF, and G-CSF, but not in that of Ep.

To determine whether or not inhibitory effects of calmodulin antagonists on the growth of colonies are caused by a direct action on hematopoietic progenitor cells, we used the human myeloid leukemic cell line, KG-1, as the homogenous target cells. KG-1 cells are known to respond to GM-CSF by forming colonies in an in vitro clonal assay. The colony formation of KG-1 cells induced by GM-CSF was dose dependently inhibited by the calmodulin antagonists (Fig 5), thereby supporting the proposal that inhibitory effects of the calmodulin antagonists on the growth of colonies are a direct action on hematopoietic progenitor cells. In biochemical analyses, calmodulin-dependent kinase activity was shown to be present in KG-1 cells and to be eluted at a different fraction from protein kinase C (Fig 1). The calmodulin antagonists led to an inhibition of calmodulin-dependent kinase activities partially purified in vitro, and the inhibitory effects on calmodulin-dependent kinase activities correlated relatively well with the potency in inhibiting colony formation of KG-1 cells. Thus, the biologic effects of calmodulin antagonists are apparently mediated through the calmodulin-mediated molecular pathway. Although it is likely that the pharmacologic effects of calmodulin antagonists are a result of actions on several calmodulin-mediated pathways, our findings do show that calmodulin antagonists inhibit calmodulin-dependent kinase activity. Moreover, these results support the notion that calmodulin-dependent kinase activity may exert a pivotal role in the growth of hematopoietic progenitor cells.

We reported that protein kinase C, a Ca²⁺/phospholipid-dependent protein kinase, may be involved in the proliferation of human CFU-C but not in that of CFU-E. We also have evidence to support the possibility that protein kinase C is involved in the growth mechanism of hematopoietic progenitor cells stimulated by rhIL-3 plus rhEp, or rhGM-CSF plus rhEp (data not shown). Therefore, these two Ca²⁺-dependent processes, protein kinase C and calmodulin-mediated system, may be implicated as regulatory components for signal transduction of IL-3, GM-CSF, and G-CSF, but not for that of Ep. Other investigators found that intracellular mechanisms involved in the action of hematopoietic growth factors are mediated through protein kinase C systems. It has also been reported that tyrosine kinase is associated with the mechanism of action of hematopoietic growth factors. Several different mechanisms appear to be involved in the mechanism of hematopoiesis. Evidence has
yet to be obtained for molecular interactions between Ca\(^{2+}\)-dependent and tyrosine kinase-mediated systems.

The data described herein provide the first evidence that calmodulin apparently is a common regulatory component in the mechanism of growth of human hematopoietic progenitor cells. Because large numbers of hematopoietic progenitor cells are difficult to obtain for detailed biochemical examinations, biopharmacologic approaches will facilitate studies to elucidate the molecular mechanisms of hematopoiesis.

ACKNOWLEDGMENT
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REFERENCES


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