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Whetton, A.D., Heyworth, C.M., Nicholls, S.E. et al. (4 more authors) (1994)
Cytokine-mediated protein kinase C activation is a signal for lineage determination in bipotential granulocyte macrophage colony-forming cells. *Journal of Cell Biology*, 125 (3). pp. 651-659. ISSN 0021-9525

<https://doi.org/10.1083/jcb.125.3.651>

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Cytokine-mediated Protein Kinase C Activation Is a Signal for Lineage Determination in Bipotential Granulocyte Macrophage Colony-forming Cells

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Abstract. Granulocyte macrophage colony-forming cells (GM-CFC) have the potential to develop into either macrophages and/or neutrophils. With a highly enriched population of these cells we have found that although GM-CFC are equally responsive to macrophage colony stimulating factor (M-CSF) and stem cell factor (SCF) in terms of DNA synthesis, M-CSF stimulated the development of colonies containing macrophages in soft gel assays, while SCF promoted neutrophilic colony formation. When SCF and M-CSF were combined, mainly macrophage development was stimulated both in soft agar colony-forming assays and liquid cultures. An analysis of some potential signaling mechanisms associated with cytokine-mediated devel-

opmental decisions in GM-CFC revealed that M-CSF, but not SCF, was able to chronically stimulate phosphatidylcholine breakdown and diacylglycerol production, indicating that protein kinase C (PKC) may be involved in the action of M-CSF. Furthermore, M-CSF, but not SCF, can increase the levels of PKC α (PKC α) expression and stimulate the translocation of PKC α to the nucleus. When the PKC inhibitor, calphostin C, was added to GM-CFC cultured in M-CSF then predominantly neutrophils were produced, conversely PKC activators added with SCF stimulated macrophage development. The data indicate a role for PKC in M-CSF-stimulated macrophage development from GM-CFC.

A number of cytokines have now been identified which can promote hemopoiesis *in vivo* and *in vitro*, and several of them share a common subset of target cells (Whetton and Dexter, 1989; Metcalf, 1991). For example, granulocyte/macrophage colony-forming cells (GM-CFC)¹, have been shown to proliferate and develop into colonies containing mature neutrophils and/or macrophages in soft agar, in response to the growth factors granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage-CSF (GM-CSF), interleukin 3 (IL-3), macrophage-CSF (M-CSF), interleukin 6 (IL-6), and stem cell factor (SCF) (Metcalf, 1984, 1989; Williams et al., 1987; Cook et al., 1989; Met-

calf and Nicola, 1991; Heyworth et al., 1993). There are, however, differences as well as similarities in the effects of these agents on GM-CFC. For example, GM-CFC treated with M-CSF proliferate and develop into colonies that consist predominantly of macrophages and G-CSF, IL-6, and SCF all promote the formation of neutrophilic colonies.

The proliferative response elicited by this diverse range of hemopoietic growth factors in GM-CFC is presumably based on some common molecular mechanism activated by the occupation of their respective receptors. Thus, it is interesting to note that there is at least one common cellular signaling event (increased intracellular pH [Cook et al., 1989]) activated by a range of colony-stimulating factors, which appears to be required for the survival of GM-CFC, *i.e.*, with suppression of programmed cell death (Rajotte et al., 1992). What is not clear, however, is how growth factors such as SCF, M-CSF, and G-CSF elicit such varied developmental responses in GM-CFC and how the signals are related to receptor occupancy. The specific receptors for the colony-stimulating factors GM-CSF, IL-3, M-CSF, SCF, G-CSF, and IL-6 (Witte, 1990; Brizzi et al., 1991; Nicola and Metcalf, 1991; Hara and Miyajima, 1992) have now

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1. *Abbreviations used in this paper:* 1,2DAG, 1,2-diacylglycerol; BrdU, 5-bromo-2'-deoxyuridine; G-CSF, granulocyte colony-stimulating factor; GM-CFC, granulocyte/macrophage colony-forming cells; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-3, Interleukin 3; M-CSF, macrophage colony-stimulating factor; PC, phosphatidylcholine; PI, propidium iodide; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; SCF, stem cell factor.

been molecularly cloned and the derived amino acid sequences exhibit no discernible similarities between the various cytokines which stimulate neutrophilic development or between those that stimulate macrophage development. The SCF receptor (c-kit), for example, is a member of the type III (platelet-derived growth factor) protein tyrosine kinase family, and has significant sequence homology with the M-CSF receptor (Bernstein et al., 1990; Witte, 1990). Nonetheless, it is clear that the developmental response elicited by these two cytokines is quite different (Metcalf and Nicola, 1991; Heyworth et al., 1992).

The activated SCF receptor can interact with target proteins such as the p85 subunit of phosphatidylinositol-3-kinase (PI3K) (Reith et al., 1991; Rottapel et al., 1991; Lev et al., 1992), and phosphoinositidase C- γ (although this enzyme is not activated to any great extent by SCF [Lev et al., 1991]) and possibly the ras GTPase-activating protein (Herbst et al., 1991). M-CSF can also stimulate the phosphorylation of PI3K and a ras GTPase-activating protein (in fibroblasts which have been transfected with the M-CSF receptor) (Sherr, 1991). M-CSF has also been shown to activate the hydrolysis of phosphatidylcholine to form 1,2-diacylglycerol, which leads to the activation of protein kinase C (PKC). Phorbol ester-mediated direct activation of PKC can promote macrophage development in a highly enriched population of GM-CFC and also in some leukemic cell lines (Lotem and Sachs, 1979; Liebermann et al., 1981; Griffin et al., 1985; Collins, 1987; Heyworth et al., 1993). To date, there is no evidence that SCF or G-CSF can activate PKC (Imamura et al., 1990; Veis and Hamilton, 1991).

Unfortunately no hemopoietic cell line model exists where the differential developmental effects of SCF and M-CSF can be investigated. Furthermore, most of the studies performed to date on hemopoietic growth factor-stimulated signaling events have concentrated solely on the common effects of hemopoietic growth factors i.e., those which induce DNA synthesis and the agonist stimulated signals which may be associated with a specific developmental response in hemopoietic stem, and progenitor cells have received little attention. We have, therefore, investigated the possible second messenger pathways and the role of PKC activation/inhibition in the response of GM-CFC to two growth factors which bind to related receptors yet elicit distinctive developmental effects on hemopoietic cells. The work presented describes one of the first biochemical mechanisms for a developmental decision in primary cultures of normal hemopoietic progenitor cells.

Materials and Methods

Enrichment of GM-CFC

GM-CFC from normal murine bone marrow were obtained by elutriation centrifugation as described (Williams et al., 1987; Cook et al., 1989). In soft gel assays of the elutriated cells there were few, if any, clusters present (the mean colony size in M-CSF, IL-3, and GM-CSF was over 800 cells [Heyworth et al., 1992]).

Colony-forming Assays

Soft agar colony-forming assays were performed as described previously (Sponcer et al., 1986). Colonies were picked out at random from agar plates for morphological analysis by cytospin preparation. Cytospin preparations of both liquid culture and colony samples were stained with May-

Grunwald-Giemsa stain. Colony morphology was also assessed by staining whole colonies *in situ* using the combined specific (positive for neutrophils) and nonspecific (positive for monocytes) esterase stain. Limiting dilution assays were also performed in some experiments, GM-CFC were plated in microtiter wells in a total volume of 0.25 ml (to obtain about 1 cell/well). Wells were then examined visually and those containing only a single cell scored after being incubated in fully humidified conditions in an atmosphere of 5% CO₂ in air. Colonies (>50 cells) were counted at seven days.

In liquid culture GM-CFC were cultured in 20% (vol/vol) fetal calf serum in Iscove's medium plus other growth factors (Heyworth et al., 1992). Sources and specific activities of growth factors were as previously detailed (Heyworth et al., 1992).

Assessment of Cellular Proliferation

Proliferating cells were identified as described previously (Cook et al., 1989), using a cell proliferation assay kit purchased from Amersham International (Bucks, UK). After incubating the cells in Iscove's medium plus the appropriate growth factors, 5-bromo-2'-deoxyuridine (BrdU) and 5-fluoro-2'-deoxyuridine for 16 h at 37°C, cells were washed and cytospin preparations were prepared. These were probed with anti-BrdU as previously described (Cook et al., 1989). [³H]thymidine incorporation was assayed as previously described (Cook et al., 1989).

Measurement of Cellular Signaling Events

Measurements of growth factor-stimulated signaling events were carried out as described previously (Owen et al., 1993). In all cases cells (1×10^6 /ml) were labeled overnight at 37°C in Fischers medium supplemented with IL-3 (5 U/ml) and 1% (vol/vol) fetal calf serum (to maintain cell viability without stimulating proliferation, this treatment had no effect on the developmental potential of the GM-CFC) before washing free of growth factor, resuspending in Fischer's medium containing 0.1% (wt/vol) bovine serum albumin, and incubating for 6 h before stimulation as required.

Confocal Microscopy

Cells were incubated in Fischer's medium supplemented with 0.1% (wt/vol) bovine serum albumin (Boehringer Mannheim, Mannheim, Germany) for 4 h at 37°C, in 5% CO₂ in air before a 2-h incubation in the presence of either 20 U/ml M-CSF or 250 U/ml SCF or no added growth stimulus. Rabbit antibodies (purchased from GIBCO BRL, Gaithersburg, MD) raised against specific peptide sequences were used to probe for PKC isoforms α , δ , ϵ , and ζ . The antibody specific for PKC β_2 isoform was kindly provided by Dr. Peter Parker (Imperial Cancer Research Fund, London, UK). Secondary FITC-conjugated sheep anti-rabbit IgG antibody was purchased from The Binding Site Ltd. (Birmingham, UK). Slide preparations were made using a Shandon cytospin (500 rpm, 5 min) (Shandon Inc., Pittsburgh, PA), fixed for 10 min in ice cold methanol, and incubated with 20 μ l primary antibody for 30 min, and then washed in PBS for 10 min. Samples were then blotted dry before being incubated with 20 μ l of secondary antibody for 30 min, and washed in PBS for 30 min. Nuclei were counterstained with propidium iodide (PI) for 1-2 min and fading of fluorescence was retarded with 2.4% DABCO (BDH Chemicals Ltd., Dagenham, UK) in 80% glycerol (Johnson et al., 1982). Stained cells were analyzed using an MRC 500 instrument (BioRad Labs., Hercules, CA).

Results

SCF and M-CSF Act upon a Common Target Cell Population in Elutriated Cells Highly Enriched for GM-CFC

The majority of the cells elutriated proliferate in response to IL-3, GM-CSF, and M-CSF (Cook et al., 1989; Heyworth et al., 1992). SCF and M-CSF both stimulate a large increase in [³H]thymidine incorporation in the elutriated GM-CFC, but when added together no additive increase in [³H]thymidine incorporation was observed (Fig. 1). Also there was no difference in the M-CSF or SCF effect on GM-CFC in the presence of G-CSF: neither cytokine had either an additive or synergistic effect when added with G-CSF

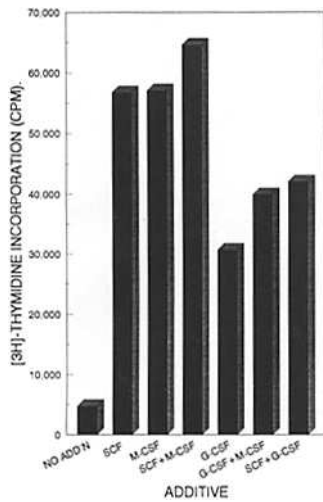


Figure 1. The effect of SCF (250 ng/ml), G-CSF (5,000 U/ml), and M-CSF (20 U/ml) on [³H]thymidine incorporation in elutriated GM-CFC. Results shown are from one typical experiment of three. The standard deviation of the mean did not exceed 10% of the mean value in any of the data points shown.

(Fig. 1). [³H]thymidine suicide assays (Table I) show that greater than 95% of the colony-forming cells which respond to M-CSF were stimulated to enter the cell cycle by SCF. Similarly, incubation of the cells with M-CSF in the presence of [³H]thymidine renders cells unable to form colonies in the presence of SCF, M-CSF, or IL-3 (Table I). Furthermore, M-CSF, SCF, and M-CSF plus SCF all stimulate DNA synthesis in 82% of the elutriated cells as assessed using anti-BrdU antibodies (Table I). Liquid cultures of elutriated cells with either M-CSF or SCF exhibited no decrease in cell viability (which remained >97%) over 3 d in culture, in other words, both cytokines were not required to maintain all the cells in culture, only one was required (in the absence of the appropriate cytokine <1% of the cells were viable after 72 h).

The SCF- and M-CSF-stimulated increase in DNA synthesis therefore occurs in a common target cell population and both these cytokines elicit a similar proliferative response within those cells.

SCF and M-CSF Have Differential Developmental Effects on Elutriated GM-CFC

The effect of SCF and M-CSF, respectively, on the types of colonies that are formed is markedly different (Table II A). M-CSF promotes the development of colonies which consist of predominantly macrophages (M ϕ), while SCF stimulates neutrophilic (N) colony formation (Tables II, A and B). This was confirmed in two ways, by taking cytopspins of individual colonies, and by using an in situ colony staining technique. Both techniques gave similar results. We next determined the effect on colony morphology of SCF + M-CSF; although no more colonies were seen than in M-CSF alone, predominantly M ϕ colonies (>99%) with only occasional (<1%) N/M ϕ colonies (Tables II, A and B) were formed. Similarly in liquid cultures, M-CSF and SCF promoted macrophage and neutrophilic development, respectively (Table III). When M-CSF was present with SCF there was a twofold increase in the numbers of cells formed over 7 d (see Heyworth et al., 1992) compared to that seen with M-CSF alone; however, the vast majority of cells formed were macrophages (see Table III) and there was no evidence of SCF-stimulated neutrophilic development in these cultures at any stage. As the majority of the cells respond to M-CSF, the data suggest that the neutrophilic stimulus elicited by SCF is apparently suppressed in the presence of M-CSF. These data encouraged us to investigate early biochemical events stimulated by these growth factors in an attempt to distinguish between common signals which may be associated with proliferation and DNA synthesis and noncommon signals involved in lineage restricted development and the dominance of the M-CSF developmental signal.

A Common Signal Associated with Proliferation Is Elicited by M-CSF and SCF

We have previously demonstrated that M-CSF stimulates the amiloride-sensitive Na⁺/H⁺ antiport in GM-CFC leading to an increase in intracellular pH (Cook et al., 1989). SCF (250

Table I. M-CSF and SCF Effects on the Proliferative Status of Elutriated Cells

Proliferating cells (% cells which are bromo deoxyuridine antibody positive)	Growth factor present in preincubation	Growth factor present in soft gel colony-forming assay after preincubation \pm [³ H]thymidine	Colonies formed (% control incubations where [³ H]thymidine was omitted from the preincubation)
82 \pm 7	M-CSF	M-CSF	0
	M-CSF	SCF	4 \pm 2
	M-CSF	IL-3	0
82 \pm 1	SCF	M-CSF	2 \pm 2
	SCF	SCF	0
	SCF	IL-3	0
82 \pm 1	SCF + M-CSF	M-CSF	2 \pm 1
	SCF + M-CSF	SCF	0
	SCF + M-CSF	IL-3	3 \pm 3

For [³H]thymidine suicide assays elutriated cells were preincubated for 8 h at 37°C in the absence of growth factors (to reduce the number of cells in cycle, a preincubation which did not decrease cell viability, or plating efficiency, which was 35% in the presence of M-CSF and 21% in the presence of SCF) and then preincubated with the growth factors shown above (see second column), in serum free Iscove's medium \pm 100 μ Ci/ml [³H]thymidine at a concentration of 2×10^5 cells per ml for 16 h. This overnight incubation does not decrease the plating efficiency of the cells (Kan et al., 1991). Cells were then washed and taken for soft gel assays in the presence of the cytokines shown in the third column. Results shown are the mean \pm SEM of three experiments and are expressed as the percent control assays in which [³H]thymidine was omitted from the initial preincubation. Plating efficiency in the control incubations which had not been treated with [³H]thymidine was similar to that observed in Table II.

For immunocytochemical labeling of proliferating cells the initial preincubation was followed by incubation in serum free Iscove's medium plus other additives (see Materials and Methods). In control incubations without cytokines <10% of the cells were stained positively. Concentrations of growth factors employed were: M-CSF, 20 U/ml; SCF, 250 ng/ml; IL-3, 100 U/ml.

Table II A. The Effect of PKC Activators and PKC Inhibitors on the Development of GM-CFC in Soft Agar Colony-forming Assays

	Colonies formed (per 200 cells plated)	Colony type formed			
		Blast	M	G	G/M
		%	%	%	%
IL-3	78 ± 8	0	15	3	82
SCF	44 ± 8	3	1	88	8
SCF + M-CSF	92 ± 6	0	97	0	3
SCF + TPA	46 ± 6	0	79	5	15
SCF + BRYO	42 ± 5	0	90	5	5
SCF + TPA + CAL	42 ± 2	3	54	37	6
SCF + BRYO + CAL	37 ± 8	3	50	47	3
M-CSF	87 ± 6	0	98	0	2
M-CSF + CAL (50 ng/ml)	94 ± 9	0	23	69	8
M-CSF + CAL (100 ng/ml)	93 ± 9	0	10	84	6
Calphostin (50 ng/ml)	0 ± 0	—	—	—	—
Calphostin (100 ng/ml)	0 ± 0	—	—	—	—

M, macrophage colonies; GM, granulocyte/macrophage colonies; G, granulocyte colonies; Blast, those colonies made up of blast cells. The concentrations of additives were: bryostatin, 10^{-8} M; TPA, 100 ng/ml; M-CSF, 20 U/ml; SCF, 250 ng/ml. BRYO, bryostatin; CAL, calphostin, which unless otherwise stated was present at a final concentration of 100 ng/ml. Colony formation is shown as total colonies per 200 cells plated and also as a percentage of the number of colonies formed in the presence of an optimal concentration of IL-3 (100 U/ml) and show the mean ± SEM from three experiments. We have previously shown that TPA or bryostatin alone do not stimulate colony formation.

Table II B. The Effect of Agents Which Potentiate the Activity of PKC on Colonies Formed from GM-CFC

Addition	Colony type formed		
	Macrophage	Granulocyte	Granulocyte/Macrophage
	%	%	%
M-CSF	99	0	1
M-CSF + CAL	41	29	30
SCF	0	90	10
SCF + TPA	80	3	17
SCF + BRYO	92	0	8
SCF + TPA + CAL	16	44	40
SCF + M-CSF	81	4	15

The effect of agents which potentiate the activity of PKC on colonies formed from GM-CFC, using in situ colony staining for combined esterases in soft agar assays. M, macrophage colonies; GM, granulocyte/macrophage colonies; G, granulocyte colonies. The concentrations of additives were: bryostatin, 10^{-8} M; TPA, 100 ng/ml; M-CSF, 20 U/ml; SCF, 250 ng/ml. BRYO, bryostatin; CAL, calphostin, which was used at a concentration of 100 ng/ml.

ng/ml) stimulated an amiloride-sensitive rise of 0.09 ± 0.02 U from a resting pH_i of 6.94 ± 0.05 (mean ± SD, $n = 4$) while M-CSF (20 U/ml) gave a rise of 0.11 ± 0.02 (mean ± SD, $n = 6$). There was no observed additive effect of M-CSF and SCF at the concentrations shown. These results show that some intracellular signaling events are commonly activated in SCF- and M-CSF-treated GM-CFC.

Growth Factor Stimulation of Phospholipid Breakdown Pathways in GM-CFC

The hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate the second messengers inositol-1,4,5-trisphosphate

Table III. The Effect of Combinations of CSFs on the Types of Cells Formed from GM-CFC in Liquid Culture

Growth factor added	Fold increase in cell number	Cell type			
		Blast	Mφ	Early granulocyte	Late granulocyte
		%	%	%	%
M-CSF	25	1	98	1	0
SCF	20	20	5	22	53
M-CSF + SCF	40	1	99	0	0

Growth factor concentrations were as shown for Fig. 1 A. The results shown are for cells incubated for 7 d, seeded at an initial density of 2×10^4 /ml, and are from one typical experiment of three. Mφ, macrophage.

and sn-1,2-diacylglycerol (1,2DAG) occurs in many cells (Berridge et al., 1982). The results shown in Table IV indicate that neither M-CSF nor SCF stimulate an increase in inositol phosphates in GM-CFC. M-CSF has previously been reported to stimulate phosphatidylcholine (PC) hydrolysis leading to the generation of 1,2DAG. An acute, transient increase in [3 H]1,2DAG was observed in M-CSF-treated cells 15 s after stimulation ($133 \pm 8\%$ of control, mean ± SEM, $n = 4$) with a return to control levels after 1 min, but no such increase was observed in SCF-treated cells (15 s–5 min). However, the commitment of the GM-CFC to differentiation occurs over a period of several hours, rather than minutes (see Metcalf, 1984 and Whetton, A. D., and S. E. Nicholls, unpublished observations) and we therefore ascertained whether there was a more persistent effect of M-CSF on the levels of 1,2DAG (Table IV). M-CSF stimulated a significant increase in [3 H]1,2DAG levels over a 2-h time course, whereas SCF had no such effect.

Measurement of the water soluble products of [3 H]PC breakdown in GM-CFC illustrate that M-CSF but not SCF is able to stimulate a significant increase in [3 H]phosphocholine levels. Glycerophosphocholine and choline levels were unaffected by either M-CSF (suggesting phospholipase C-mediated hydrolysis of PC, as previously reported, [Imamura et al., 1990]) or SCF. These data infer that there is a long term effect of M-CSF on PC hydrolysis, as has been reported for some other growth factors (Cook and Wakelam, 1992). This may contribute to the observed increase in 1,2DAG levels and clearly distinguishes the activity of M-CSF from that of SCF.

Expression and Activation of Protein Kinase C Isoforms by M-CSF and SCF in GM-CFC

Since 1,2DAG is the physiological activator of PKC, the above results suggest that M-CSF, but not SCF, can activate PKC. Thus, the activation of PKC may have a role to play in determining the dominance of the M-CSF signal. Indeed, M-CSF has been shown to activate PKC in other cell types (Imamura et al., 1990; Veis and Hamilton, 1991). However, direct activity assays or Western analysis of subcellular fraction-associated PKC was not possible in highly enriched GM-CFC due to the low numbers of cells obtained from the elutriation procedure. Because of this we used confocal microscopy to determine whether or not stimulation with M-CSF would lead to alterations in the patterns of PKC isoform expression and distribution. First, we ascertained the

Table IV. The Effect of Cytokines on Lipid Hydrolysis in GM-CFC

Additive	Time	[³ H]inositol phosphate (percentage of control)	[³ H]1,2 DAG (percentage of control)	Total [³ H]choline levels (percentage of control)	Total [³ H]choline phosphate levels (percentage of control)
	<i>min</i>				
M-CSF (20 U/ml)	30	91 ± 2	106 ± 9	106 ± 5	126 ± 11
M-CSF (20 U/ml)	60	ND	128 ± 12	103 ± 6	126 ± 15
M-CSF (20 U/ml)	120	ND	149 ± 10	97 ± 12	115 ± 1
SCF (250 ng/ml)	30	97 ± 13	103 ± 7	92 ± 11	92 ± 12
SCF (250 ng/ml)	60	ND	111 ± 9	95 ± 4	88 ± 8
SCF (250 ng/ml)	120	ND	103 ± 7	100 ± 18	102 ± 7

As a measure of inositol lipid hydrolysis [³H]inositol phosphate levels were measured in [³H]inositol-loaded GM-CFC. Results shown are from a 30-min incubation. [³H]1,2 DAG levels were determined in cells loaded to isotopic equilibrium with [³H]glycerol which after loading were washed and left without cytokines for 4 h prior to challenge with cytokines. [³H]choline and [³H]choline phosphate levels were assessed in cells loaded to isotopic equilibrium with [³H]choline. The results shown are the mean ± SEM of at least three observations. Incubations with fluoroalumininate (2 μM AlCl₃, 0.5 mM NaF) for 30 min gave a 155 ± 10% increase (mean ± SEM, n = 4) in [³H]inositol phosphate levels in parallel incubations to those reported in the table above. Further time points on [³H]inositol phosphate levels were not assessed as the presence of lithium interferes with cellular metabolism and can effect cellular viability.

levels of the different PKC isoforms expressed in GM-CFC. There was a high degree of expression of PKC isoforms β (which apparently consisted predominantly of the β₂ isoform), α and ζ, whereas the PKC δ and ε isoforms were only present in relatively low levels in the elutriated cells. It is important to state that the distribution of the PKC isoforms was uniform throughout >95% of the cells (Fig. 2).

We next determined the effect of chronic treatment of the GM-CFC with M-CSF and SCF, respectively, on the levels and intracellular location of PKC isoforms. Neither M-CSF nor SCF affected the levels or the subcellular localization of PKC β₁, PKC ζ, PKC δ, or ε over a 2-h period. The level of fluorescence (mean integrated fluorescence intensity per cell) detected using the PKCβ₂ antibody was unaltered by

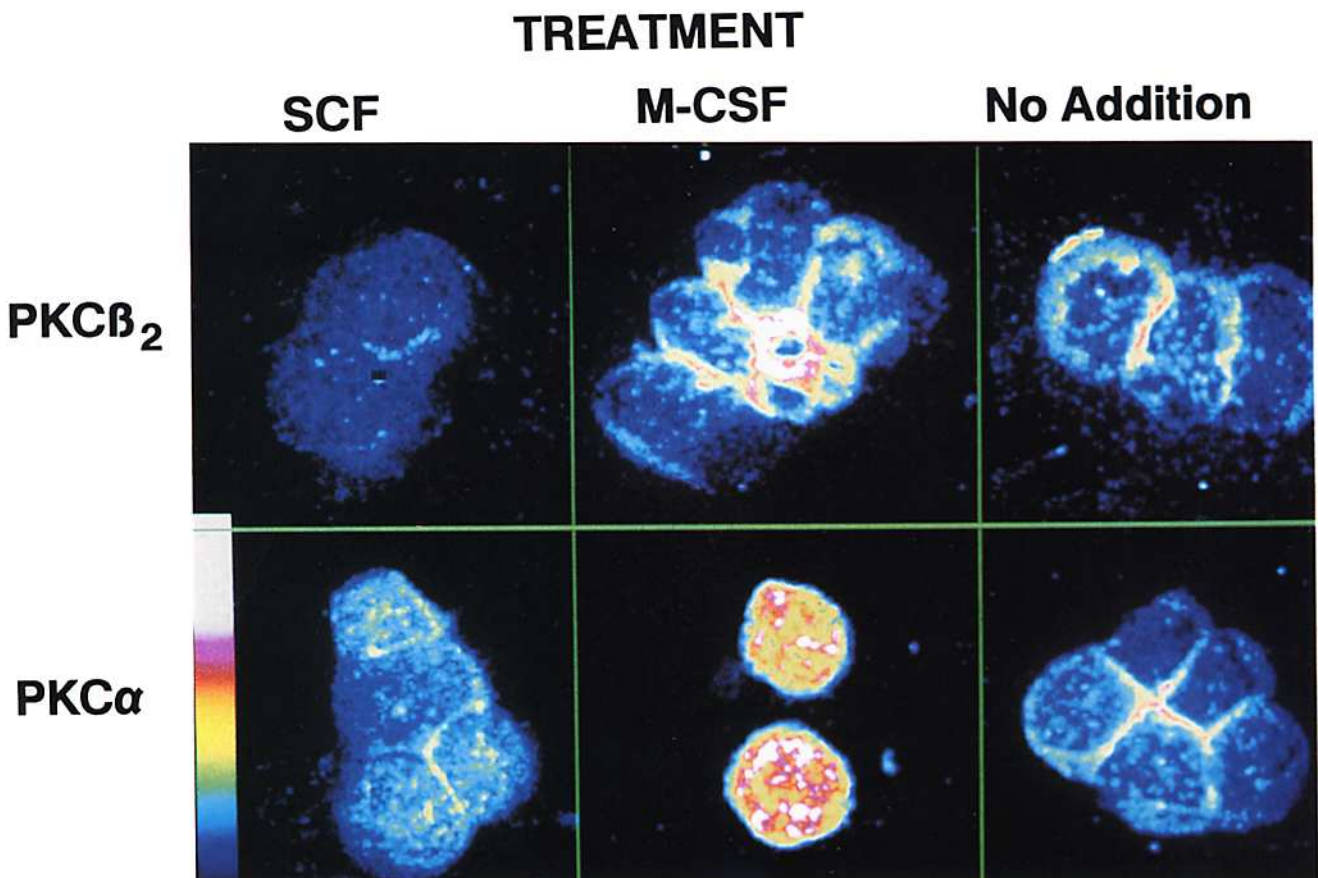


Figure 2. The effect of a 2-h incubation with SCF and M-CSF, respectively, on the levels and distribution of PKC isoforms in elutriated GM-CFC. The fields shown contain cells which were typical of >95% of those observed.

incubation with M-CSF for 2 h, however there was a marked decline in fluorescence seen in SCF-treated cells (Fig. 2). The subcellular location of the PKC β_2 in control cells and SCF-treated cells was predominantly cytosolic, as was the case for M-CSF-treated cells.

When elutriated cells were probed with a PKC α -specific antibody, marked differences were discernible in the effects of SCF and M-CSF. M-CSF stimulated an increase in the level of expression of PKC α and also the translocation of this protein from the cytosol to the nucleus (optical sectioning through the cell revealed that this was not a translocation to the perinuclear region). SCF, on the other hand, did not affect either the location or the level of PKC α within the cell. It is important to note that these effects were observed in the majority of cells studied (>95%).

When the PKC inhibitor calphostin C (100 ng/ml) was added to GM-CFC at the same time as M-CSF we found that there was a marked inhibition of M-CSF-stimulated PKC α translocation to the nucleus.

Activation of PKC Biases GM-CFC Development to the Macrophage Lineage

We have previously reported that activators of PKC, such as TPA or bryostatin, when used in combination with a neutrophil stimulus, G-CSF, can promote macrophage development from GM-CFC in both soft gel and liquid cultures (Heyworth et al., 1993). This is also the case when PKC activators are added with SCF (see Table II B). Furthermore GM-CFC cultured with TPA or bryostatin develop into macrophages (Heyworth et al., 1993).

IL-4 has been shown to activate inositol lipid hydrolysis and PKC in monocytes and B lymphocytes (Finney et al., 1990; Harnett et al., 1991; Arruda and Ho, 1992) and to stimulate the translocation of PKC to the nucleus (as reported above for M-CSF-treated GM-CFC) (Arruda and Ho, 1992). Moreover, IL-4 has also been reported to increase the levels of colony formation when used in combination with G-CSF (Broxmeyer et al., 1988; Rennick et al., 1989; Vellenga et al., 1990; Jansen et al., 1991), an effect similar to that seen with TPA or bryostatin (Heyworth et al., 1992). In GM-CFC, IL-4 (100 ng/ml) stimulated an increase in total inositol phosphate levels over a 30-min incubation in the presence of lithium chloride ($144 \pm 11\%$ compared to control samples, mean \pm SEM, $n = 3$). Furthermore, IL-4 can also increase 1,2DAG levels in GM-CFC: incubations of 30, 60, and 120 min led to $120 \pm 23\%$, $148 \pm 12\%$, and $189 \pm 33\%$ increases in the 1,2DAG levels, respectively (mean \pm SEM, $n = 3$).

IL-4 alone (10 or 100 ng/ml) did not stimulate colony development in soft agar assays of GM-CFC and modestly inhibited colony formation when combined with M-CSF (Fig. 3). When added with SCF, however, although there was no significant effect on the number of colonies formed, there was a marked switch to M ϕ colonies (see Fig. 3). In limiting dilution assays where one cell/well was present SCF (250 ng/ml) stimulated the development of 85% N colonies with 15% N/M ϕ colonies whereas SCF plus IL-4 (100 ng/ml) gave 45% M ϕ colonies and 55% N colonies. The plating efficiency seen with SCF was unaltered by the addition of IL-4. These results show that even cytokines which have no apparent colony-stimulating activity, but which can activate specific signaling pathways, may influence the types of colo-

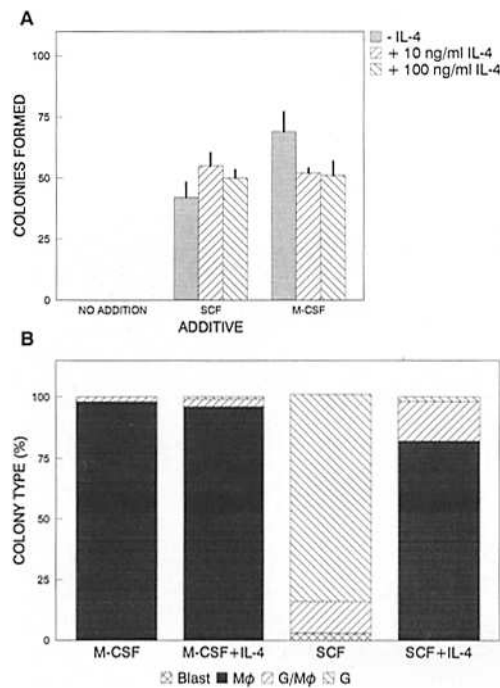


Figure 3. The effect of Interleukin-4 on the development of GM-CFC. (A) The effect of IL-4 on the colony formation in the presence of either M-CSF or SCF. Optimal concentrations for colony formation were used for all growth factor additions (see Table II). Colonies formed were counted after 7 d. Results shown are the mean \pm SEM of three experiments. (B) The percentage of each colony type was assessed by picking out >40 colonies in each experiment and staining the cells as described. Results are taken from one typical experiment of three. G/M ϕ denotes granulocyte/macrophage colonies, M ϕ denotes macrophage colonies, and G denotes granulocytic colonies.

nies formed from GM-CFC in response to other cytokines or growth factors.

Inhibition of PKC Activity Biases the Development of GM-CFC Towards the Neutrophilic Lineage

PKC activation stimulates the preferential development of macrophages from GM-CFC; does the inhibition of PKC stimulate increased neutrophilic development? To test this, we used the PKC inhibitor calphostin, which has the advantage of binding to the regulatory domain of PKC thereby having a greater degree of specificity than other inhibitors (Tamaoki and Nakano, 1990). There was a marked effect of calphostin on the types of colonies formed in the presence of M-CSF in that predominantly neutrophilic colonies were formed, although the total number and size of the colonies remained unchanged (see Table II A).

Similar results were also observed in limiting dilution assays (1 cell/well [Heyworth et al., 1993]). The plating efficiency was unaltered by calphostin, but the types of colonies formed were markedly different: M-CSF gave 90% M ϕ colonies plus 10% N/M ϕ colonies, whereas M-CSF + calphostin (100 ng/ml) gave 52% N colonies, 33% N/M ϕ colonies, and 15% M ϕ colonies. Cultures of GM-CFC grown in SCF + TPA or SCF + bryostatin (which consist of mainly M ϕ colonies) (see Table II B), are replaced by a mixture of N, N/M ϕ , and M ϕ colonies in the presence of calphostin.

The differential effects of calphostin on the levels of N, N/M ϕ , and M ϕ colonies formed may be associated with the differential modes of activation of PKC employed (i.e., direct activation by TPA versus agonist-mediated stimulation by the action of M-CSF on the cells).

In limiting dilution assays, results similar to those described in Tables II, A and B were obtained. In SCF + bryostatin, 78% M ϕ and 22% N/M ϕ colonies were formed; the further addition of calphostin (50 ng/ml) affected a change in colony morphology to 12% M ϕ , 34% N/M ϕ , and 54% N colonies. When calphostin was added to colony-forming assays where only one cell/well was present with SCF + TPA there was a similar shift in the types of colonies formed. PKC inhibition by calphostin can influence lineage choice, promoting neutrophilic colony development from GM-CFC.

Discussion

Granulocyte macrophage colony-forming cells have the potential to develop into either macrophages or neutrophils and it has been known for some time that cytokines can influence the developmental decisions within GM-CFC. Experiments with parent, daughter, and granddaughter cells of these bipotential progenitor cells have clearly shown that the regulation of lineage commitment is, at least in part, extrinsic: the growth factors in which the cells are cultured can influence the type of mature cells formed (Metcalf, 1984). From these daughter cell experiments, it has also been established that the loss of the bipotential nature of GM-CFC that occurs in the presence of growth factors is not an immediate event (occurring within minutes or even hours) and also that the commitment (when it occurs) is essentially irreversible.

To assess the mechanisms that influence the proliferation and development of granulocyte macrophage progenitor cells, we have used enriched murine GM-CFC as a target cell population that can develop into either neutrophils or macrophages, depending on the culture conditions employed (Williams et al., 1987; Cook et al., 1989). While there is obviously a degree of heterogeneity in GM-CFC isolated from bone marrow and within the cell population prepared, we and others have shown that the population is enriched for GM-CFC (Williams et al., 1987; Cook et al., 1989) and, furthermore, that most of the cells respond to SCF and M-CSF, respectively. For example the majority of the cells proliferate when either M-CSF or SCF is added to cultures containing elutriated cells (see Table I); M-CSF and SCF have a markedly less than additive effect on the proliferation of elutriated cells or colony formation (Table I and Fig. 1); the same effect of SCF and M-CSF, respectively, on PKC isozyme distribution is seen in >95% of the cells. Furthermore, M-CSF stimulated colony formation from about 43% of the cells plated (Table I), and SCF did not significantly increase this figure when added to plating assays with M-CSF. We have therefore compared the biochemical events stimulated by these growth factors in an attempt to distinguish between common signals which may be associated with a proliferative response and more specific signals which may be linked to lineage commitment and development to either neutrophils or macrophages (see Tables II, A and B).

Both cytokines stimulated a similar increase in pH_i within the elutriated cells, indicating that some of the signals

elicited by these cytokines are held in common and may be associated with general housekeeping functions and (eventually) DNA synthesis. However, M-CSF has previously been reported to stimulate PC breakdown to generate the second messenger diacylglycerol (Imamura et al., 1990). We observed increases in 1,2DAG and phosphocholine levels, but not choline levels. These results are consistent with a chronic activation of a phospholipase C-mediated PC hydrolysis stimulated by M-CSF (SCF had no such effects). Classically a rapid activation of inositol lipid hydrolysis can be followed by a prolonged increase in PC hydrolysis and 1,2 DAG accumulation. We have confirmed here the results obtained on M-CSF-stimulated monocytes where there is an increase in 1,2 DAG levels derived from PC hydrolysis with no concomitant inositol lipid hydrolysis (Whetton et al., 1988; Imamura et al., 1990). Thus we have identified one example of a biochemical event stimulated by M-CSF, which is not activated by SCF, and which therefore may be involved in the differential developmental effects of M-CSF compared to SCF.

Because diacylglycerol is the physiological activator of the classical PKC isoforms (Nishizuka, 1992), a prediction from this data is that M-CSF, but not SCF, would stimulate PKC in GM-CFC. This was found to be the case: confocal microscopy experiments have shown that M-CSF does indeed have a profound effect on the levels and subcellular localization of one of the classical isoforms of PKC, PKC α , but not on others. SCF, on the other hand, has little effect on the localization of any isoform within the GM-CFC. This then raises the question as to whether or not the translocated PKC α can influence the development of GM-CFC, presumably via modification of transcription. In this respect it has been previously demonstrated that PKC may be important in hemopoietic cell development. For example, certain leukemic cell lines develop into macrophages under the influence of PKC activators (Lotem and Sachs, 1979; Liebermann et al., 1981; Collins, 1987); M-CSF is known to activate PKC (Imamura et al., 1990; Veis and Hamilton, 1991) while there is no evidence to suggest that SCF or G-CSF can activate PKC; phorbol esters can influence the development of myeloid progenitor cells leading to macrophage development (Griffin et al., 1985; Heyworth et al., 1993). Interestingly, while SCF did not translocate PKC β_2 after 2 h, this isozyme was almost completely downregulated by SCF. Thus the ability of this cytokine to promote neutrophil development may be mediated by its downregulation of PKC β_2 . Support for this proposal comes indirectly from other studies with phorbol esters. TPA promotes macrophage development in myeloid progenitor cells (Bunce et al., 1990) and several authors have shown that PKC β is more susceptible to downregulation by TPA than PKC α (Kiley et al., 1990).

Perhaps more compelling evidence of a role for PKC in macrophage development comes from our studies using activators and inhibitors of this enzyme. In this way we have now demonstrated that chronic activation of PKC does indeed bias the development of GM-CFC towards the macrophage lineage, while inhibition of PKC leads to the preferential production of neutrophils even in the presence of a potent macrophage stimulus such as M-CSF. Our data infer that the PKC isozyme activated during macrophage lineage development is PKC α and the isozyme inhibited, possibly by downregulation, to determine neutrophil development is PKC β_2 . Furthermore this finding may well be of physiological

significance particularly with respect to cytokines such as IL-4. Although there is some evidence to suggest that IL-4 can stimulate PKC α translocation to the nucleus in GM-CFC (Whetton, A. D., S. E. Nicholls, and J. M. Lord, unpublished observation) plainly IL-4 may potentiate the developmental decisions taken by these bipotential cells via some other mechanism. What is important, however, is that IL-4 can potentiate developmental decisions in GM-CFC without stimulating colony formation directly. In other words, the local production of IL-4 within the environment of the bone marrow may be able to influence the lineage options taken by the adjacent GM-CFC. Much emphasis has been placed on the synergy between cytokines leading to growth promotion; we now suggest that developmental potentiation is another critical role for cytokines (which, like IL-4 on GM-CFC, need not have any CSF activity per se).

At present no inhibitors for specific PKC isoforms are available and further studies to discriminate between the roles of these kinases are difficult to perform. One way to approach this, however, is by overexpressing the appropriate isoform and where this has been performed. PKC α was shown to be associated with neural induction and competence in *Xenopus* (Otte and Moon, 1992). There may therefore be an important role for PKC in commitment decisions in a number of different tissues.

The biological effects of overexpression of constitutively activated PKC isoforms (see Otte and Moon, 1992) in GM-CFC, however, are difficult to perform since GM-CFC are a transit cell population and cannot be delayed in their development to mature cells for any great length of time. Here we have provided biochemical and cell biological data that implicates PKC α activation and translocation in growth factor-mediated lineage restriction decisions in a highly enriched population of bipotential committed hemopoietic progenitor cells.

We wish to thank Stella Pearson for her expert technical assistance and Dr. Gerald Johnson for his help and advice on confocal microscopy. We wish to thank Dr. D. Metcalf for his comments on this work.

This work was supported by the Leukaemia Research Fund. T. M. Dexter is a Gibb Fellow of, and his work is supported by the Cancer Research Campaign. J. M. Lord is a Royal Society University Research Fellow.

Received for publication 5 November 1993 and in revised form 31 January 1994.

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