

Alterations in arachidonic acid metabolism in mouse mast cells induced to undergo maturation in vitro in response to stem cell factor

James M. Samet, PhD,^a Alfred N. Fonteh, PhD,^a Stephen J. Galli, MD,^c Mindy Tsai, PhD,^c Mary Beth Fasano, MD,^d and Floyd H. Chilton, PhD^{a, b}
Winston-Salem, N.C., and Cambridge, Mass.

We studied arachidonic acid (AA) metabolism during the maturation of bone marrow-derived cultured mast cells (BMCMCs) into mast cells with phenotypic characteristics, which were more similar to those of connective tissue-type mast cells. BMCMCs were maintained in medium containing 100 ng/ml recombinant rat stem cell factor (SCF) for 1 to 6 weeks. After 3 to 4 weeks in SCF, BMCMCs acquired many phenotypic characteristics of maturation, including enlarged size, numerous electron-dense cytoplasmic granules, and a 50-fold elevation in histamine content. Maintenance in SCF for 6 weeks did not significantly alter the amounts or species of eicosanoids that were produced by BMCMCs stimulated with calcium ionophore A23187. However, SCF-treated mast cells released 2.6 ± 0.13 times more free AA and accumulated 6.4 ± 1.0 times higher levels of intracellular free AA than did immature BMCMCs not exposed to SCF. There was no increase in the mobilization of other fatty acids (e.g., linoleic or oleic acid), indicating specificity for AA. Moreover, there were no differences between the 5-lipoxygenase activities of SCF-treated or untreated cells, as assayed in cell homogenates prepared by nitrogen cavitation. Although the total AA content in SCF-treated cells was significantly elevated, the distribution of AA in phospholipid and neutral lipid classes was not altered by SCF treatment. Total phospholipase (PL)_{A2} activity increased $85\% \pm 11.5\%$ in SCF-treated cells. In homogenates of immature BMCMCs, $51.0\% \pm 13.7\%$ of the PL_{A2} activity was inhibited by 0.5 mmol/L dithiothreitol, whereas the same concentration of dithiothreitol caused only a $2.2\% \pm 10.7\%$ reduction in the PL_{A2} activity in homogenates of SCF-treated BMCMCs ($p \leq 0.05$, $n = 4$). These findings suggest that SCF treatment induces a dithiothreitol-resistant PL_{A2} and that this PL_{A2} may contribute to the mobilization of AA that is not further metabolized to eicosanoids. (J Allergy Clin Immunol 1996;97:1329-41.)

Key words: Mast cell, stem cell factor, phospholipase A₂, arachidonic acid, eicosanoids

The generation of chemical mediators by mast cells and the subsequent effects of these compounds on cells and tissues are pivotal events in the

pathogenesis of inflammatory and allergic reactions.¹⁻⁹ Mast cells produce a wide variety of inflammatory mediators, including lipid mediators such as arachidonic acid (AA) and its metabolites (eicosanoids).^{3, 6, 9} Morphologic and histochemical studies demonstrate the presence of multiple mast cell phenotypes within a single organism.^{4, 9} At least some of the phenotypic differences among various mast cell populations can reflect the extent of maturation exhibited by these cells.⁴ For example, mouse bone marrow-derived cultured mast cells (BMCMCs), which are generated in medium containing IL-3 synthesize and store relatively small amounts of histamine, incorporate sulfur 35-labeled sulfate predominantly into chondroitin sulfates, and exhibit an alcian blue-positive, safranin-negative histochemical phenotype.¹⁰ However,

From ^aSection on Pulmonary and Critical Care Medicine and ^bDepartment of Biochemistry, Bowman Gray School of Medicine, Winston-Salem; ^cDepartments of Pathology, Harvard Medical School and Beth Israel Hospital, Cambridge; and ^dSection on Infectious Diseases, Bowman Gray School of Medicine, Winston-Salem.

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Reprint requests: Floyd H. Chilton, PhD, Section on Pulmonary and Critical Care Medicine, Bowman Gray School of Medicine, Winston-Salem, NC 27154.

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Abbreviations used

AA:	Arachidonic acid
BMCMCs:	Bone marrow–derived cultured mast cells
CTMCs:	Connective tissue–type mast cells
DTT:	Dithiothreitol
GC/MS:	Gas chromatography/mass spectrometry
$^2\text{H}_8$:	Octadeuterated
$^2\text{H}_4$:	Tetradecuterated
HBSS:	Hanks' balanced salt solution
HPLC:	High-performance liquid chromatography
LT:	Leukotriene
PG:	Prostaglandin
PL:	Phospholipase
SCF:	Stem cell factor
TX:	Thromboxane

after such IL-3–derived BMCMCs are maintained for 4 to 6 weeks in medium supplemented with recombinant rat stem cell factor (SCF), the ligand for the SCF receptor encoded by *c-kit*, they undergo maturation as assessed by morphology, a striking increase in their content of cell-associated histamine, a marked increase in their ability to incorporate ^{35}S -sulfate into heparin proteoglycan, and the acquisition of a safranin-positive histochemical phenotype.¹⁰ In addition, they develop a mouse mast cell protease messenger RNA phenotype that is more similar to that of connective tissue–type mast cells (CTMCs).¹¹ Therefore prolonged maintenance of IL-3–derived BMCMCs in SCF induces the cells to express phenotypic characteristics, which are more similar to those of mouse CTMCs. On the other hand, BMCMCs maintained in SCF do not become fully mature CTMCs, at least as judged by their histamine content and histochemical characteristics.¹⁰

The effects of long-term SCF culture on the ability of BMCMCs to release or metabolize AA have not previously been reported. There are at least three biochemical steps in the release and metabolism of AA, which could influence the ability of BMCMCs to synthesize eicosanoids. First, the metabolic fate of AA can be influenced by the availability of AA in certain phospholipid pools. The content of AA varies considerably among phospholipid classes and subclasses in cells that produce different eicosanoid profiles.¹²⁻¹⁴ Recent studies in our laboratory indicate that AA is

mobilized from several phosphoglyceride pools in BMCMCs undergoing immunologic activation.¹⁵ Further, these studies suggest that AA derived from these separate pools may be metabolized to different classes of AA products.¹⁵

A second factor that may influence eicosanoid profiles in BMCMCs is the types of phospholipase (PL) A_2 isozymes, which are present within the cell. Two calcium-dependent PLA_2 enzymes that may be important in mobilizing AA from cellular phospholipids have been identified in mammalian cells. Group I and II PLA_2 (s PLA_2) are low-molecular-weight (14 kd) enzymes found in extracellular fluids. Group II s PLA_2 is known to be released from several cells and is found in inflammatory exudates.¹⁶⁻¹⁹ This activity is resistant to acidic pH, is sensitive to reducing agents such as dithiothreitol (DTT), and catalyzes the nonspecific release of several saturated and unsaturated fatty acids. A second form of PLA_2 , with a relatively large molecular weight (110 kd), has been found in the cytosolic fraction of several cell types.²⁰⁻²³ This cytosolic PLA_2 (c PLA_2) requires micromolar calcium, is sensitive to low pH, and is resistant to DTT. Substrate specificity studies indicate that c PLA_2 preferentially hydrolyzes AA from the *sn*-2 position of phospholipids.^{18, 19, 21} Murakami et al.²⁴ demonstrated the presence of three distinct PLA_2 activities in IL-3–derived BMCMCs, including a group II PLA_2 , c PLA_2 , and a phosphatidylserine-specific PLA_2 . At present, little is known about the role of different PLA_2 enzymes in the synthesis of eicosanoids by BMCMCs or other cell types.

A third factor that may influence eicosanoid profiles in BMCMCs is the activities of enzymes distal to PLA_2 in the eicosanoid cascade. Malaviya et al.²⁵ have recently demonstrated that immunologic activation of IL-3–derived BMCMCs results in the reversible translocation and activation of 5-lipoxygenase. However, little is known about the influence of BMCMC maturation on activation or translocation of 5-lipoxygenase. Similarly, it is not known whether SCF-induced maturation can influence the induction of cyclooxygenase (cyclooxygenase I or cyclooxygenase II) in BMCMCs.

To better understand the factors that regulate AA metabolism in mast cells, we attempted to determine whether IL-3–derived BMCMCs, which had been induced to undergo phenotypic changes associated with maturation by long-term culture in SCF,¹⁰ also exhibited changes in their AA precursor pools, in the enzymes that release or metabolize AA, or in the amount of AA or eicosanoids released on stimulation of the cells.

METHODS

Materials

Tritiated AA-labeled *Escherichia coli* (10 mCi/mmol) and [5,6,8,9,11,12,14,15-³H]AA (76 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). Calcium ionophore A23187, fatty acid-free human serum albumin, and diisopropyl fluorophosphate were purchased from Sigma Chemical Co. (St. Louis, Mo.). All high-performance liquid chromatography (HPLC) solvents were obtained from Fisher Scientific (Charlotte, N.C.). Racemic 13-hydroxy-9,11-octadecadienoic acid and deuterated AA and eicosanoids were purchased from Cayman (Ann Arbor, Mich.). Unlabeled fatty acids and phospholipids were purchased from Avanti (Birmingham, Ala.). All derivatization reagents were bought from Pierce Chemical Co. (Rockford, Ill.). Thin-layer chromatography plates were obtained from Analtech (Newark, Del.). Preparative silica columns were obtained from Baker (Phillipsburgh, N.J.). All tissue culture reagents such as Hanks' balanced salt solution (HBSS) and tissue culture media were purchased from Gibco (Grand Island, N.Y.). Mouse IL-3 supplement was obtained from Collaborative Research (Bedford, Mass.). *E. coli*-derived recombinant rat SCF¹⁶⁴ (SCF), which represents essentially the entire extracellular ligand domain of the growth factor,¹⁰ was provided by AMGEN, Inc. (Thousand Oaks, Calif.).

Cell culture

Normal mouse bone marrow cells were obtained from CBA/J mouse femurs as described previously and cultured for 3 weeks in a medium consisting of RPMI-1640 with 10% heat-inactivated fetal calf serum, 5 mmol/L 2-mercaptoethanol, 100 U/ml penicillin and streptomycin, and 50 mg/ml gentamicin (growth medium), supplemented with 5% mouse IL-3.²⁶ Cells were fed twice per week and placed in new flasks in order to remove adherent cells. This procedure routinely yielded 95% mast cells, with a viability of greater than 90%.²⁶

To induce further maturation of BMCMCs,^{10,11} some BMCMCs were then placed, for 0 to 42 days, in growth medium without IL-3 but containing 100 ng/ml SCF. Phenotypic changes indicative of maturation were evident by 28 to 42 days of culture in SCF (see Results). The cells were provided with fresh growth medium containing SCF three times per week. Electron micrographs were prepared from 100 nm sections of glutaraldehyde-fixed cells embedded in epoxy resin and imaged by using a Philips 400 TEM electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).²⁷ Cells were routinely tested for maturation by staining with alcian blue, safranin O, or toluidine blue.²⁸

Stimulation of BMCMCs and extraction of glycerolipids

BMCMCs were washed once with 0.25 mg/ml human serum albumin in HBSS and then twice with HBSS. Cells were resuspended in HBSS with 1 mmol/L calcium

TABLE I. Phenotypic changes in BMCMCs treated with SCF for 28 days

	Untreated BMCMCs	SCF-treated BMCMCs
Granule content (no. of cytoplasmic granules)	+	++++
Staining		
Safranin O	—	++
Alcian blue	+	+++
Toluidine blue	+	+++
Histamine content (nmol/10 ⁶ cells)	14.4	675
Histamine release (% of total)		
Control	16.6	13.2
Stimulated†	29.3	34.8
Protein content (mg/10 ⁶ cells)	20.1 ± 0.94	36.4 ± 2.1*
Lipid phosphorus (mg/10 ⁶ cells)	10.6 ± 0.36	19.4 ± 1.0*

A minus sign indicates that a particular characteristic cannot be observed. The number of plus signs indicates the degree that a particular characteristic can be observed.

*Significant difference from untreated BMCMCs ($p < 0.05$, $n \geq 3$).

†A23187 (1 μ mol/L $\times$ 5 minutes).

at a concentration of 1.0×10^6 /ml and preincubated at 37° C for 5 minutes before stimulation with 1 μ mol/L (final concentration) calcium ionophore A23187 for 5 minutes. Cells were then immediately pelleted by centrifugation ($250 \times g$ for 5 minutes). ²H₈AA (≥ 98 atom % D; Cayman, Ann Arbor, Mich.) (100 ng) was added to cell pellets as an internal standard for quantitation of free fatty acids. Lipids were extracted from the cell pellets by the method of Bligh and Dyer.²⁹ Supernatant fluids were added to 4 volumes of ethanol containing an internal standard mixture consisting of 10 ng each of ²H₄ prostaglandin (PG)D₂, ²H₄PGE₂, ²H₄PGF_{2 α} , ²H₄ thromboxane (TX)B₂, ²H₄ leukotriene (LT)B₄ and 100 ng of ²H₈AA. For the analysis of AA distribution in phospholipid classes of BMCMCs, cellular lipids were extracted by using the method of Bligh and Dyer.²⁹ Glycerolipid classes were then separated by normal phase HPLC as described below.

PLA₂ and 5-lipoxygenase assay

BMCMCs were washed twice in HBSS and placed in HBSS containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L NaF, and 5 mmol/L diisopropyl fluorophosphate for 10 minutes on ice. The cells were then washed, resuspended in HBSS/EDTA/NaF at 2.5×10^6 cells/ml, and cavitated twice under N₂ at 750 psi for 15 minutes in a cell cavitation vessel. Unbroken cells were removed by centrifugation (twice at 500 g for 5 minutes). Because the amount of protein per cell increased as the cells differentiated in response to SCF

treatment (Table I), PLA₂ activity was always assayed by using an amount of protein equivalent to a predetermined number of cells (2.5×10^5 cells) in a total volume of 400 μ l containing 50 mmol/L Tris HCl (pH 8.5), 50 mmol/L NaCl, 10 ml of tritiated AA-labeled *E. coli* suspension and 10 mmol/L CaCl₂ in HBSS/EDTA/NaF. In experiments in which DTT was used, this reagent was added to the disrupted cell preparations for 15 minutes on ice before the assay was started. All PLA₂ reactions were started by the addition of CaCl₂ and substrate and allowed to proceed for 60 minutes at 37° C. The reaction was stopped by extracting the lipids by the method of Bligh and Dyer.²⁹ Solvents were removed under a stream of N₂, and lipids were chromatographed as described below.

5-Lipoxygenase activity was assayed in a predetermined number of cells (1 to 1.5×10^6 cells), essentially as described by Coffey et al.³⁰ Briefly, aliquots of cell cavities were diluted to 600 μ l in HBSS/EDTA/NaF, and final concentrations of 100 mmol/L Tris HCl (pH 7.5), 1.8 mmol/L adenosine triphosphate, 5 mmol/L CaCl₂, and 1 mmol/L DTT were added. The reaction was initiated by the addition of a substrate mix consisting of 0.1 μ Ci tritiated AA, 1.67 μ mol/L unlabeled AA, and 4.5 μ mol/L 13-hydroxy-9, 11-octadecadienoic acid (final concentrations) in methanol. The reaction was incubated for 60 minutes at 37° C, after which 4 volumes of ethanol were added and the samples were dried under a stream of N₂ for analysis by HPLC as described below.

Histamine, lipid phosphorus, and protein assays

Histamine levels were measured in aliquots of supernatant fluids from stimulated and resting BMCMCs by using a commercially available iodine 125-labeled histamine radioimmunoassay kit (AMAC, Inc., Westbrook, Maine). Lipid phosphorus was assayed directly in thin-layer chromatography scrapings.³¹ BMCMC protein samples were assayed with a Coomassie blue reagent available from Bio-Rad (Richmond, Calif.).

Chromatographic techniques

Lipid extracts from BMCMCs were fractionated into neutral lipid classes (triglycerides, diglycerides, monoglycerides, and free fatty acids) on silica gel G plates with a solvent system consisting of hexane/ether/formic acid (90:60:6 vol/vol). This system was also used to separate free AA from phospholipids in lipid extracts after PLA₂ assays. Phospholipid classes were separated by using silica gel G thin-layer chromatography plates developed in chloroform/methanol/ammonium hydroxide (65:35:8). 5-Lipoxygenase assay products were fractionated by reverse-phase HPLC on a Beckman Ultrasphere ODS 5 μ m column (2 $\times$ 250 mm; Beckman Instruments, San Ramon, Calif.) with a Waters HPLC system (Waters, Milford, Mass.). Products were eluted with methanol/water/phosphoric acid (55:45:0.02 vol/vol, pH adjusted to 5.0 with ammonium hydroxide) at a flow

rate of 0.3 ml/min. The methanol composition was increased to 100% in a linear gradient over 60 minutes.³² Quantities were calculated from the ratio of the area of each LT peak to the PGB₂ internal standard with previously calibrated standard curves.

Neutral lipid and phospholipid classes were fractionated by HPLC on a Beckman Ultrasphere silica 5 μ m (4.6 $\times$ 250 mm) column by using a mobile phase consisting of hexane/2-propanol/ethanol/25 mmol/L phosphate buffer pH 7.4/acetic acid (490:367:100:30:0.2 vol/vol) (solvent A) and hexane/2-propanol/ethanol/25 mmol/L phosphate buffer pH 7.4/acetic acid (490:367:100:50:0.6 vol/vol) (solvent B) pumped at 1 ml/min. Solvents were stirred overnight and filtered before use. After 5 minutes, the phosphate buffer content was increased from 3% to 5% over a 5-minute period, and phospholipids were eluted by maintaining this solvent composition for 30 minutes.²⁶ UV absorbance in column effluent was monitored at 206 nm with a Waters 490E UV detector. Fractions corresponding to neutral lipids or individual phospholipid classes were then pooled for analysis of fatty acid content by gas chromatography/mass spectrometry (GC/MS).

Fatty acid and eicosanoid determination by GC/MS

Samples were prepared for GC/MS analysis of fatty acids and prostanoids by sequential derivatization as described previously.^{32, 33} Methoxime derivatives of ketone groups were prepared by incubation with methoxyamine-HCl in pyridine for 12 hours at 30° C. Carboxyl groups were converted to pentafluorobenzyl esters with pentafluorobenzyl bromide (20% in acetonitrile) in diisopropylethylamine (20% in acetonitrile) for 20 minutes at 40° C, and hydroxyl groups were silylated by reaction with N,O-bis(trimethylsilyl)trifluoroacetamide for 15 minutes at 40° C. Carboxylate anions of LTB₄ (m/z 479), ²H₄LTB₄ (m/z 483), PGD₂ (m/z 424), ²H₄PGD₂ (m/z 428), TXB₂ (m/z 614), and ²H₄TXB₂ (m/z 618) were analyzed by negative ion chemical ionization GC/MS.³³

Fatty acids esterified in glycerolipid classes isolated by HPLC were hydrolyzed with 2 mol/L KOH in methanol/water (75:25 vol/vol) containing 100 ng ²H₈AA/sample as an internal standard for 30 minutes at 60° C. Samples were then neutralized with HCl, and free fatty acids were extracted twice with ethyl ether. Solvents were removed under N₂, and fatty acids were converted to pentafluorobenzyl esters as described previously for GC/MS analysis. For analysis of intracellular free fatty acids, cellular lipid extracts were loaded in hexane on preparative Bakerbond (J. T. Baker, Phillipsburgh, N.J.) silica columns. Fatty acids were then eluted with hexane/ether 1:1 vol/vol, and solvents were removed with a stream of N₂ and converted to pentafluorobenzyl esters as described previously for GC/MS analysis. All samples were analyzed on a Hewlett-Packard 5890 series II gas chromatograph interfaced to a 5989A Quadrupole mass

spectrometer and a 98785A work station. Derivatized samples were analyzed by means of negative ion chemical ionization GC/MS. Carboxylate anions of AA (m/z 303) and ^2H_8AA (m/z 311) were monitored.³³

Statistical analyses

Data are expressed as means $\pm$ SEM. Data were analyzed by using paired or unpaired Student's *t* tests or analysis of variance, followed by Bonferroni-corrected *t* tests for post hoc analysis.³⁴

RESULTS

Characterization of IL-3-dependent and SCF-treated BMCMCs

Electron microscopy of 3-week-old IL-3-derived mouse BMCMCs showed that these cells contained relatively few cytoplasmic granules and that these granules were immature with large electron-lucent areas (Fig. 1, *A*). After 3 to 4 weeks of culture in SCF, the BMCMCs contained increased numbers of cytoplasmic granules, and these appeared much more mature than those in IL-3-derived BMCMCs that had not been treated with SCF. Notably, many of the cytoplasmic granules in the SCF-treated BMCMCs had abundant electron-dense content (Fig. 1, *B*).

In accord with the ultrastructural findings, SCF-treated BMCMCs stained strongly with dyes that bind to cytoplasmic granules, such as alcian blue, toluidine blue, and safranin (Table I). In contrast, IL-3-derived BMCMCs that had not been treated with SCF stained weakly with alcian blue or toluidine blue and failed to stain with safranin (Table I). Thus the IL-3-derived BMCMCs treated with recombinant rat SCF (100 ng/ml) for 4 to 6 weeks in this study, like those in our previous study in which SCF was used at a concentration of 50 ng/ml, exhibited histochemical changes indicative of maturation toward CTMC-like mouse mast cells.¹⁰

In addition to changes in structure and histochemistry, SCF treatment induced a marked increase in the total histamine content of BMCMCs. For example, there was a 50-fold increase in the histamine content of BMCMCs after 28 days of treatment with SCF (Table I). However, the percent release of histamine by SCF-treated cells, both under resting conditions and in response to stimulation with the calcium ionophore A23187, was similar to that observed in BMCMCs that were not exposed to SCF (Table I). SCF-treated cells contained 1.8 times more protein and 1.8 times more total lipid phosphorus (phospholipid content) than the untreated cells, probably in proportion to the relative sizes of the cells. However, there were no differences in the distribution of

lipid phosphorus among phospholipid classes (i.e., PE, PI, PS, and PC) between BMCMCs not treated with SCF and those treated with SCF for 42 days (data not shown). Taken together, these data confirm results of our previous study,¹⁰ indicating that in vitro treatment with SCF can induce maturation of BMCMCs.

AA metabolism during maturation of mast cells

In subsequent experiments we used GC/MS to examine the profile of eicosanoids produced by BMCMCs at various stages of maturation to determine whether the phenotypic alterations that were induced by SCF included changes in the spectrum of eicosanoids produced by these cells. Mouse BMCMCs challenged with ionophore A23187 produced primarily PGD₂, TXB₂, and LTB₄ (Fig. 2) and a small amount of LTC₄ (data not shown), whereas unstimulated BMCMCs released undetectable amounts of AA and eicosanoids (data not shown). BMCMCs treated with SCF initially showed a transient but striking reduction in their capacity to produce eicosanoids in response to A23187 challenge. Proportionate decreases were observed in the amounts of PGD₂, TXB₂, and LTB₄ released by BMCMCs, which were challenged 7 to 21 days after transfer into medium containing SCF. However, by day 42 of SCF treatment, the production of each of these eicosanoids had returned to levels that were not significantly different from those observed in BMCMCs not treated with SCF (Fig. 2). Furthermore, there were no significant differences in the ratio of 5-LO to cyclooxygenase products synthesized by BMCMCs that had not been exposed to SCF or had been matured with SCF for 42 days.

We also attempted to determine whether SCF treatment influenced the release and accumulation of free AA by BMCMCs (Fig. 2). As seen with the eicosanoids, levels of AA release by stimulated cells decreased between days 7 and 21 of treatment with SCF. However, in contrast to the results with eicosanoids, BMCMCs treated with SCF for 42 days released 2.4 ± 0.12 times more free AA when challenged than did identically stimulated BMCMCs that had not been treated with SCF (Fig. 2).

To determine whether the increased release and accumulation of AA by SCF-treated cells was specific for AA, we measured the levels of AA and other fatty acids in A23187-stimulated SCF-untreated or SCF-treated BMCMCs. Cells treated with SCF for 42 days released 302 ± 15.6 pmol of

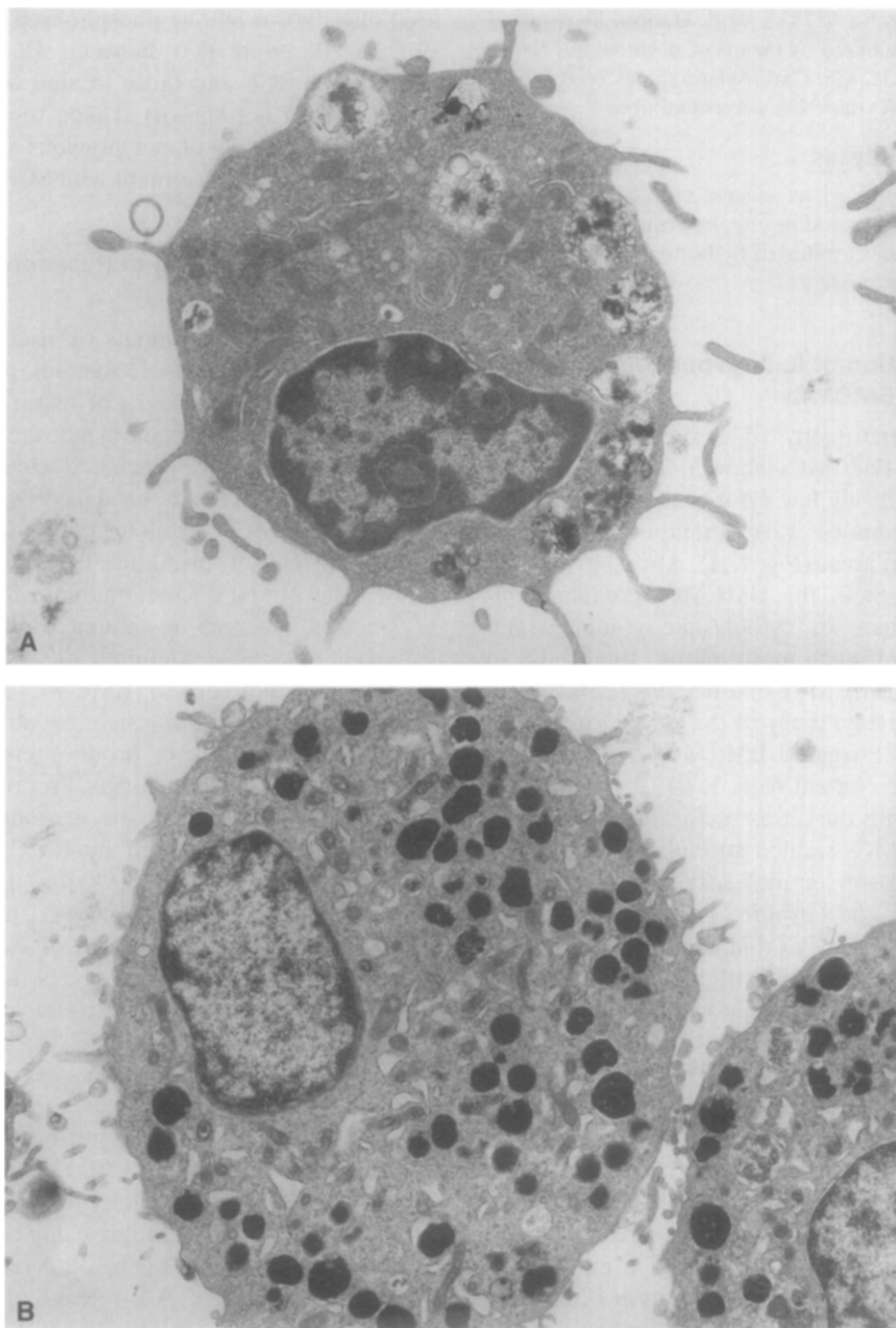


FIG. 1. Transmission electron micrographs of BMCMCs. **A**, Untreated IL-3-derived BMCMCs not maintained in SCF. Cytoplasm contains relatively few granules, which appear immature, containing a mixture of electron-dense and electron-lucent areas. **B**, BMCMCs derived in IL-3 and maintained in 100 ng/ml SCF for 4 weeks. There are numerous cytoplasmic granules, and most contain abundant, homogeneously electron-dense material. (Magnification $\times 7000$.)

AA per million cells, whereas BMCMCs that had not been treated with SCF released 124 ± 21.0 pmol per million cells (Fig. 3, *A*). The greatest increases in AA levels were seen intracellularly: there was a $6.4\text{-fold} \pm 1.0\text{-fold}$ increase in the accumulation of intracellular AA in SCF-treated

BMCMCs compared with BMCMCs that had not been exposed to SCF (Fig. 3, *B*). In contrast, either released or intracellular levels of linoleic acid produced by BMCMCs treated with SCF for 42 days were not significantly different from those found in BMCMCs that had not been treated with SCF (Fig.

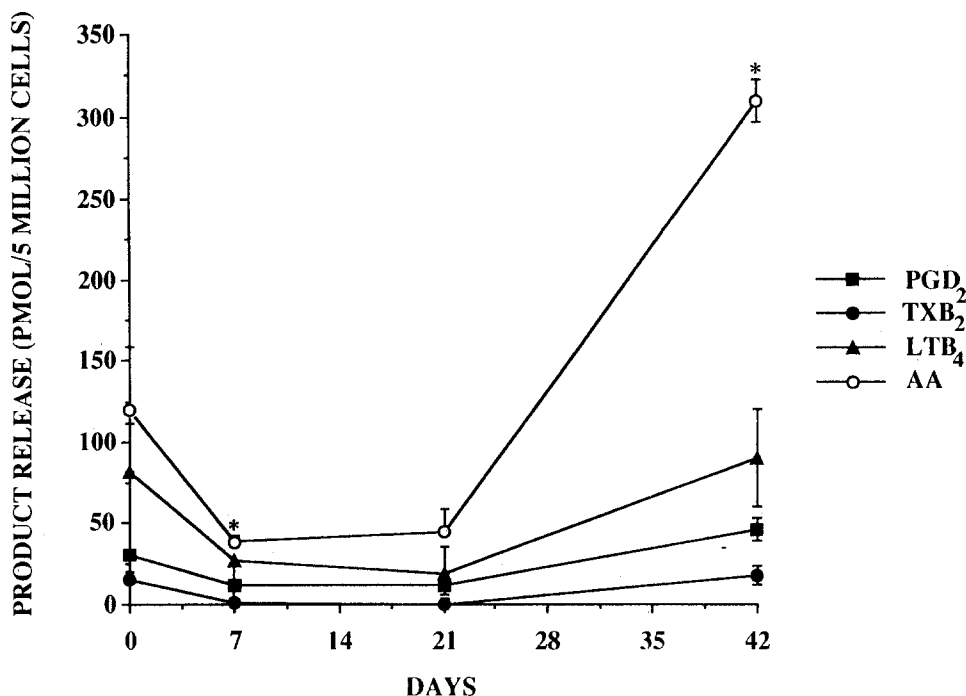


FIG. 2. Effect of culture in SCF on eicosanoid and AA production by stimulated BMCMCs. IL-3-derived mouse BMCMCs were maintained in vitro with 100 ng/ml SCF for 0, 7, 21, or 42 days and then challenged with 1 μ mol/L A23187 for 5 minutes. Eicosanoids and AA released into supernatant fluids were measured by GC/MS as described in Methods. Asterisk indicates significant difference from day 0 cells ($p < 0.05$, $n \geq 3$).

3). Similarly, treatment with SCF did not have an effect on the amounts of oleic acid mobilized by BMCMCs (data not shown). These data indicate that treatment of BMCMCs with SCF selectively increased the capacity of BMCMCs to mobilize free AA that was not further metabolized to eicosanoids.

Mechanisms of AA release in SCF-treated mast cells

Several biochemical mechanisms could account for the increased levels of free AA mobilized by SCF-treated cells. One potential explanation is that SCF treatment increased the AA content of BMCMCs. We therefore used GC/MS to examine the effect of SCF treatment on the AA content of neutral lipids and the major phospholipid classes in BMCMCs. BMCMCs treated with SCF for 42 days resulted in a 2.2-fold $\pm$ 0.2-fold increase in the total AA content of mast cell glycerolipids. Thus BMCMCs treated for 42 days contained 2.68 ± 0.25 nmol of glycerolipids per 10^6 cells compared with 1.20 ± 0.17 nmol per 10^6 cells in untreated BMCMCs ($p < 0.002$, $n = 4$). However, there were no significant changes in the relative quantities of AA contained in various lipid classes (i.e., neutral lipids, phosphatidylethanol-

amine, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine) in BMCMCs treated with SCF (Fig. 4). For example, phosphatidylethanolamine contained the bulk (45% to 60%) of AA, whereas phosphatidylcholine and phosphatidylinositol contained smaller quantities (28% to 35% and 13% to 20%, respectively) in both SCF-treated and untreated cells. These data show that although the total AA levels were higher in SCF-treated cells, the increased production of AA by SCF-treated cells was not associated with a change in the distribution of AA among glycerolipid pools within these cells.

A second possibility is that an increase in PLA₂ activity contributed to the enhanced AA release in SCF-treated cells. To assess this possibility, we disrupted equivalent numbers of SCF-treated and untreated cells by nitrogen cavitation and measured the PLA₂ activity. PLA₂ activity in BMCMCs that had not been treated with SCF was 353 ± 33.6 pmol of AA hydrolyzed per hour per 10^6 cells. There was a statistically significant 1.85-fold $\pm$ 0.12-fold increase in PLA₂ activity in BMCMCs treated with SCF for 42 days (654 ± 40.6 pmol AA hydrolyzed per hour per 10^6 cells, $p \leq 0.04$, $n = 3$). However, in contrast to our results with eicosanoid and free AA production, there was no decrease in

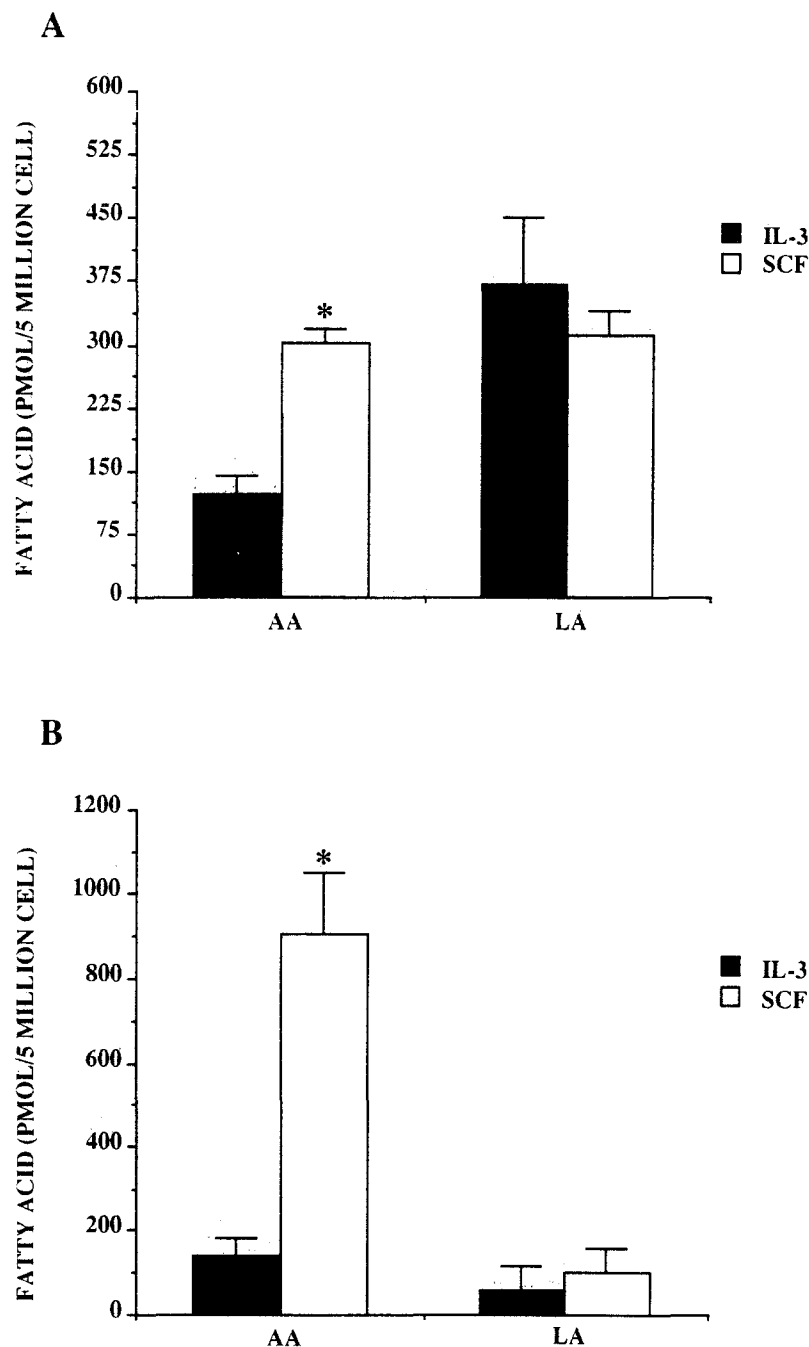


FIG. 3. Effect of culture in SCF on fatty acid release and intracellular fatty acid accumulation by BMCMCs. IL-3-derived BMCMCs or BMCMCs maintained in vitro with SCF for 42 days were stimulated with 1 μ M A23187 for 5 minutes. **A**, Extracellular AA and linoleic acid (LA). **B**, intracellular AA and linoleic acid (LA) released into supernatant fluids in IL-3-derived (IL-3) or SCF-cultured (SCF) BMCMCs. Fatty acids were measured by GC/MS as described in Methods. Asterisk indicates significant difference from IL-3-derived (IL-3) BMCMCs, ($p < 0.05$, $n = 3$).

total PLA₂ activity during the early stages of treatment with SCF (data not shown).

The increase in total PLA₂ activity in SCF-treated BMCMCs may have involved a proportional increase in the activities of all PLA₂

isozymes present within the cell or a relatively selective change in some PLA₂ isozymes. The reducing agent DTT inhibits enzymes that contain disulfide linkages, including groups I and II (secretory) PLA₂ enzymes.³² We therefore examined the

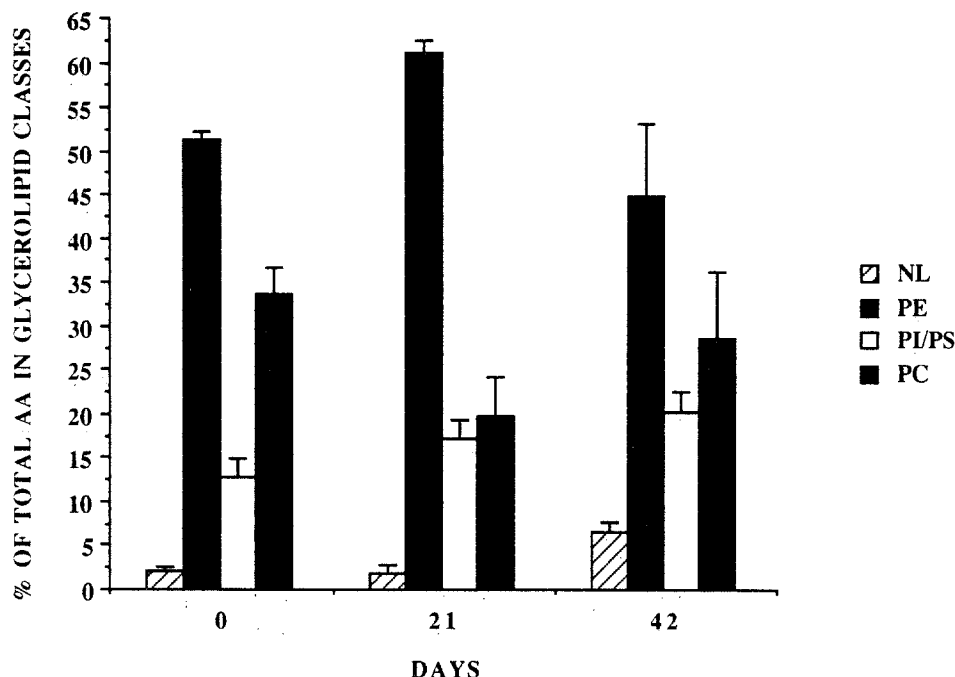


FIG. 4. Effect of culture in SCF on the distribution of AA among BMCMC glycerolipids. Glycerolipids from IL-3-derived BMCMCs maintained in SCF for 0 to 42 days were extracted by the method of Bligh and Dyer²⁹ and isolated by HPLC. Esterified fatty acids in isolated glycerolipid classes were subjected to base hydrolysis, and the resulting free fatty acids were analyzed by GC/MS. See Methods for additional details ($n = 4$). NL, neutral lipids; PE, phosphatidylethanolamine; PI/PS, phosphatidylinositol/phosphatidylserine; PC, phosphatidylcholine.

sensitivity of total PLA₂ activity to inhibition by DTT in SCF-treated or untreated BMCMC preparations. As shown in Fig. 5, BMCMCs treated with SCF for 42 days contained PLA₂ activity that was significantly more resistant to DTT inhibition, relative to the activity in BMCMCs that had not been treated with SCF. For example, 0.5 mmol/L DTT inhibited 51.0% $\pm$ 13.7% of the PLA₂ activity in SCF-untreated BMCMC cavities but only 2.2% $\pm$ 10.7% of the activity in BMCMCs that had been treated with SCF for 42 days ($p \leq 0.05$, $n = 4$). These findings suggest that treatment of BMCMCs with SCF induced an increase in the activity of DTT-resistant isozymes of PLA₂ within BMCMCs. In an effort to determine whether BMCMCs treated with SCF had elevated levels of cPLA₂ protein, cell lysates of BMCMCs at various stages of differentiation with SCF were analyzed by Western blotting with a specific anti-peptide cPLA₂ antibody. Of three separate batches of differentiated BMCMCs analyzed, one showed an increase in cPLA₂ as a result of differentiation with SCF, and two showed no change (data not shown).

One mechanism, which could explain increased levels of free AA without a concomitant increase in eicosanoid production in SCF-treated cells, is re-

duced activity of enzymes distal to AA mobilization in the eicosanoid cascade. For example, a decrease in 5-LO activity might account for the change in the ratio of AA to 5-LO products during maturation. To investigate this possibility, we assayed N₂-cavitated preparations from SCF-treated and control BMCMCs for 5-LO activity. Under optimal assay conditions, the major metabolite produced was 5-hydroxyeicosatetraenoic acid, with a small amount of LTB₄ (Fig. 6). This analysis indicated that there were no significant differences in the 5-LO activity present in SCF-treated BMCMCs as opposed to control BMCMCs that had not been treated with SCF. Moreover, there were no changes in 5-LO activity at any time during the maturation process, including the period between 7 and 21 days when the BMCMCs produced markedly reduced levels of LTB₄ (data not shown). These data indicate that treatment with SCF did not significantly affect the 5-LO activity in BMCMCs.

DISCUSSION

In this report we have investigated AA mobilization and metabolism in BMCMCs that have been induced to mature by long-term exposure to SCF in vitro. A principal finding of these studies is

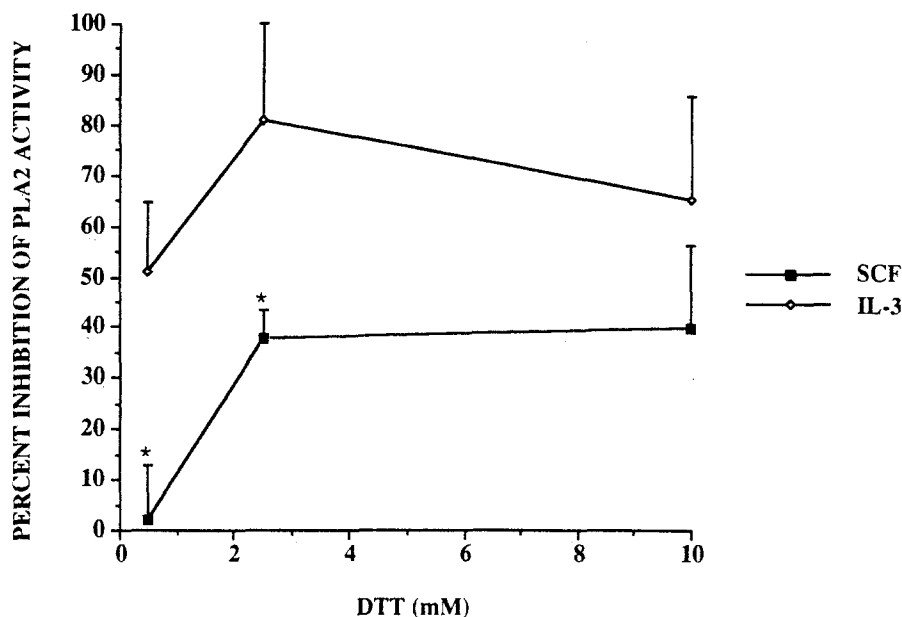


FIG. 5. Effect of culture in SCF on inhibition of PLA₂ activity by DTT. PLA₂ activity towards tritiated AA-labeled *E. coli* membranes was assayed in cellular cavities of IL-3-derived (IL-3) or SCF-cultured BMCMCs (SCF) in the presence of varying concentrations of DTT. Data are expressed as percent inhibition of activity in control samples that received no DTT. Asterisk indicates significant difference from untreated BMCMCs ($p < 0.05$, $n = 4$).

that the accumulation of free AA by BMCMCs stimulated with the calcium ionophore A23187 was significantly increased after treatment of the cells with SCF for 6 weeks. In contrast, there were no significant differences in the synthesis of 5-LO or cyclooxygenase products in BMCMCs before or after 6 weeks of treatment with SCF. Moreover, the A23187-induced mobilization of fatty acids other than AA was not enhanced in association with SCF-induced BMCMC maturation. One straightforward explanation for the increase in free AA production in the absence of an accompanying increase in eicosanoid generation would be a reduction in the activities of enzymes distal to PLA₂ (e.g., 5-LO). However, as shown in Fig. 6, BMCMC 5-LO activity did not appear to be affected by treatment of the cells with SCF.

Our data, showing the majority of the released AA to be intracellular and the majority of released linoleic acid to be largely extracellular (Fig. 3), suggest differential compartmentalization of these two fatty acids in separate lipid pools within the cell. Previous studies from our laboratory have attempted to determine whether the AA that is not further metabolized to eicosanoids and the AA that forms LTs are derived from different phospholipid sources.¹⁵ AA-containing phospholipids of BMCMCs were radiolabeled with AA such that

all major phospholipid subclasses contained different specific activities.¹⁵ By comparing the tritium to carbon 14 ratios of free AA and LTs produced by stimulated BMCMCs, we showed that free AA and AA that forms 5-LO products are probably derived from distinct AA phospholipid pools.¹⁵ One implication of these data is that the metabolic fate of AA is determined, at least in part, by its localization within these distinct lipid pools. These observations also raised the intriguing possibility that separate mechanisms control the mobilization of AA from distinct sources. In light of these previous findings, results indicating that the production of free AA, but not eicosanoids, increases in SCF-treated cells suggested two possibilities: (1) that these products are derived from separate AA pools and that SCF treatment causes a selective accumulation of AA in the pool responsible for free AA formation³⁵ and (2) that these products are derived from separate AA pools and that SCF treatment increases the cells' capacity to mobilize a pool of AA that is not further metabolized to eicosanoids.

We evaluated the possibility that the increase in AA production by SCF-treated BMCMCs is due to a shift of AA into a lipid pool, which might serve as a source of free AA. Analysis of the fatty acid content of phospholipid classes showed that the total amount of AA in the cells

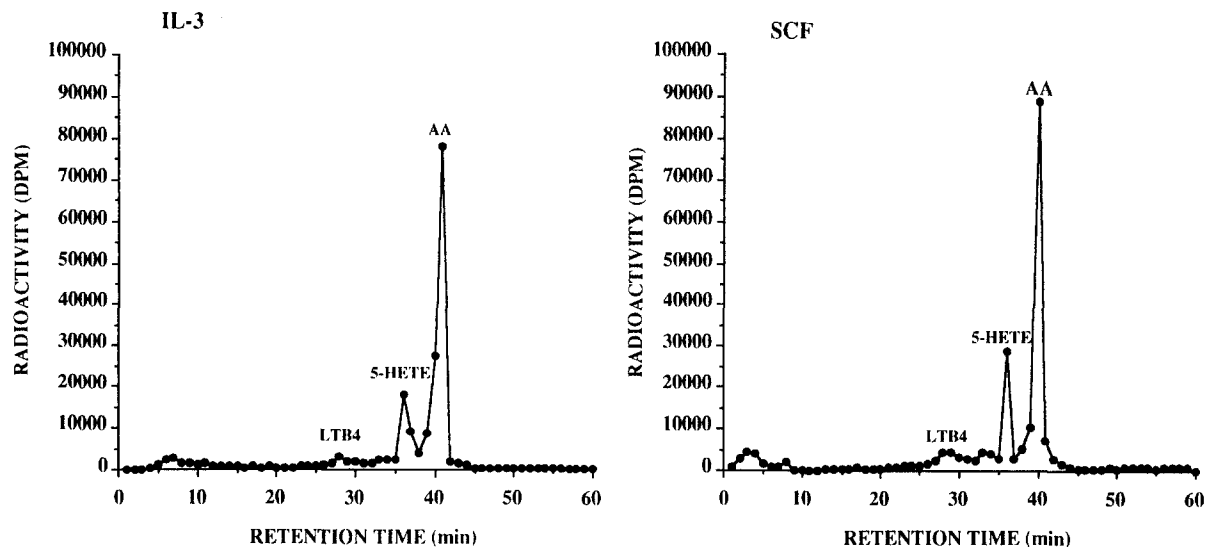


FIG. 6. Effect of culture in SCF on 5-lipoxygenase activity in BMCMCs. 5-Lipoxygenase activity was assayed in cellular cavities of IL-3-derived (*IL-3*) or SCF-cultured (*SCF*) BMCMCs. 5-Lipoxygenase products were isolated by reverse-phase HPLC and radioactivity was determined by liquid scintillation counting. *DPM*, Disintegrations per minute.

doubled, in parallel with the increases in cell size and in total protein content, which were induced by SCF treatment. However, treatment with SCF did not induce significant changes in the distribution of AA among BMCMC lipids. In agreement with this finding, SCF treatment also resulted in roughly a doubling of total phospholipid mass, yet the relative composition of phospholipid classes (as determined by lipid phosphorus) did not change in association with SCF-induced maturation. These findings suggest that the total mass of AA available in phospholipids was increased in SCF-differentiated BMCMCs. It is conceivable that SCF treatment induces a redistribution of AA within BMCMC phospholipid subclasses, a change that would not have been detected in these experiments; it is also possible that SCF induced a shift of AA between different anatomic locations within the cell. Additional studies are needed to address these issues. However, our evidence indicates that increased AA production by SCF-treated BMCMCs does not merely reflect a redistribution of AA among phospholipid classes.

A second mechanism that might account for the increased production of free AA in SCF-treated BMCMCs is the induction of an AA mobilization pathway that specifically leads to the production of free AA. One such possibility is a change in PLA₂ activity in SCF-treated BMCMCs. We found a significant increase in total PLA₂ activity in

BMCMCs treated with SCF for 6 weeks. This increase (~twofold on a per-cell basis vs BMCMCs that were not treated with SCF) paralleled the changes in total protein, total phospholipid mass, and total amount of AA in SCF-treated cells. However, SCF-treated BMCMCs also exhibited a striking increase in the resistance of the total PLA₂ activity to inhibition by DTT. These data suggest that the SCF-induced increase in BMCMC PLA₂ activity was accompanied by a change in the proportion of PLA₂ isozymes in the treated cells. It is important to note that most of the increase in total PLA₂ activity in SCF-treated BMCMCs was insensitive to DTT and coincided with an increase in the specific release of AA. Although results of Western blotting analyses for cPLA₂ were inconclusive, the data showing an increase in PLA₂ activity that was resistant to DTT treatment suggested the induction of a PLA₂ type that is distinct from sPLA₂ in BMCMCs differentiated with SCF. Additional studies are required to identify the PLA₂ isozyme increased by SCF treatment.

The roles of different PLA₂ isozymes in AA metabolism are not yet clearly defined. Work by Murakami et al.³⁶ on human umbilical vein endothelial cells indicates that both sPLA₂ and cPLA₂ contribute to the mobilization of the AA used in eicosanoid synthesis in BMCMCs. Moreover, recent findings in our laboratory support the hypothesis that sPLA₂ activity contributes some of the AA that is used for eicosanoid synthesis in BMCMCs.³²

It is possible that one PLA₂ isozyme selectively releases AA for eicosanoid metabolism, whereas another produces free AA that is not metabolized further by the cell. On the basis of our current understanding of the function of PLA₂ isotypes, as well as on the basis of data in this study, we speculate that treatment with SCF induces increased levels of cPLA₂ activity in BMCMCs and that this accounts, at least in part, for the increased production of free AA by SCF-treated BMCMCs that are stimulated by A23187.

Although free AA was once thought to be merely an intermediate in the synthesis of eicosanoids, we now recognize that it can also represent a distinct end product of cellular activation. This AA may be released by one cell type to be metabolized to eicosanoids by other cells.³⁷ In addition, AA may be a key participant in certain signal transduction pathways,^{38,39} most notably as an activator of protein kinase C^{40,41} and as a promoter of intracellular calcium mobilization^{42,43} and nucleotide binding to the plasma membrane.⁴⁴ Thus the increase in AA release and accumulation, which is observed on A23187 stimulation in BMCMCs that have been induced to mature in response to SCF, may point to an altered capacity of these cells to respond to biologic stimuli and to participate in proinflammatory cell-to-cell interactions.

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