Modulation of Erythropoiesis by Novel Human Bone Marrow Cytochrome P450-Dependent Metabolites of Arachidonic Acid

By Nader G. Abraham, Eric Feldman, John R. Falck, John D. Lutton, and Michel Laniado Schwartzman

In the hematopoietic system the adherent stromal cells produce cytokines necessary for proliferation and differentiation of hematopoietic cells. In the present study, we showed the ability of adherent stromal cells to generate novel metabolites of arachidonic acid via the NADPH-cytochrome P450-dependent monoxygenase system. These metabolites were recovered in the incubation media, suggesting their release from cells. The formation of arachidonic acid metabolites was inhibited by 7-ethoxyresorufin and SKF-525A, but not by indomethacin or BW-755C. By using two-step high-pressure liquid chromatography (HPLC), bone marrow-adherent stromal cells and incubation media showed the presence of metabolites in a peak eluted at 19 to 20 minutes. The isolated HPLC peak was used to measure its effect on colony-forming unit-erythroid (CFU-E) growth and compare it with that of synthetic cytochrome P450 arachidonate metabolites, 19- and 20-hydroxyeicosatetraenoic (HETE) acid. These bone marrow cytochrome P450 arachidonic acid metabolites at picomolar concentration potentiated erythropoietin (Epo)-induced CFU-E growth by fourfold to sixfold. Addition of 19- and 20-HETE to the bone marrow culture resulted in a potentiating effect on CFU-E number in a dose-dependent manner. 20-HETE was much more potent in stimulating CFU-E growth than 19-HETE at a similar concentration of 10^{-10} mol/L. The potentiating effect of 20-HETE resulted in a shifting to the left of the dose-response curve to Epo. To substantiate the finding of an active NADPH-dependent cytochrome P450-metabolizing system, we further examined the ability of adherent cells to metabolize exogenous pharmacologic compounds such as benzo(a)pyrene, a substrate for the heme-cytochrome P450 system, aryl hydrocarbon hydroxylase. The adherent stromal cytochrome P450 metabolizes benzo(a)pyrene at comparable levels to blood vessel endothelial cells. These novel observations underscore the importance of adherent stromal cytochrome P450 to metabolize endogenous substrates, including arachidonic acid, to compounds that may interact in a paracrine manner with Epo-dependent hematopoietic cells.

IN ADDITION TO cyclooxygenase and lipoxygenase, arachidonic acid can be metabolized by heme-cytochrome P450-dependent monoxygenases in several tissues. The microsomal cytochrome P450 enzyme comprises a family of hemoproteins that serve as the terminal acceptor in the NADPH-dependent mixed function oxidase system. This enzyme system, in the presence of NADPH and molecular oxygen, metabolizes arachidonic acid to several oxygenated metabolites including (1) four regioisomeric epoxygenosatrienoic acids (EETs) (5,6; 8,9; 11,12; 14,15), which can be hydrolyzed enzymatically by epoxide hydrolase to the corresponding diols (dioxygenosatrienoic acids [DHTs]); (2) six regioisomeric cis-, trans-conjugated hydroxytetraenoic acids (HETEs); and (3) ω and ω-1 alcohols. Several studies by our laboratory and others demonstrated that the EETs and their corresponding hydrolytic metabolites DHTs possess a wide range of biologic activities. In the hematopoietic system the presence of arachidonic acid to prostaglandin and leukotrienes are neutrophils and monocytes-macrophages, as well as several leukemic cell lines such as HEL60 and U937. Prostaglandins such as prostaglandin E2 (PGE2), mono-HETE, and leukotrienes have been implicated in the modulation of colony formation of both erythroid and myeloid progenitor cells and in differentiation processes of leukemic cell lines. For example, erythropoietin [Epo] dependen enhancement of erythropoiesis has been well documented with several arachidonic acid metabolites, such as PGE2. Although the presence of the cytochrome P450 system and its enzyme-dependent activities in bone marrow (BM) have previously been demonstrated, no information is available on cytochrome P450-dependent arachidonic acid metabolism in this tissue and the effect these novel metabolites might have on hematopoiesis.

With this in mind, the present study was undertaken to determine whether the third pathway of arachidonic acid metabolism, via cytochrome P450, is present in human BM-adherent stromal cells, and whether the metabolites derived by this enzyme system exhibit an effect(s) on hematopoiesis. The presence of an active cytochrome P450 system in these cells was further evaluated by measuring its capacity to metabolize other exogenous substrates. The cytochrome P450-arachidonate metabolites were isolated and their effect on stem cell clonal growth (colony-forming unit-erythroid [CFU-E]) was tested. Based on a similar high-pressure liquid chromatography (HPLC) elution profile of the BM-derived P450-arachidonic acid metabolite(s) and synthetic 19- and 20-HETE, the biologic activity of the synthetic compounds was also tested.

MATERIALS AND METHODS

Preparation of human BM cells. Heparinized human BM was obtained by aspiration from the posterior iliac crest of normal

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Submitted April 20, 1990; accepted May 14, 1991.

Supported in part by National Institutes of Health Grant No. AM29742 and the Harry Barowsky Clinical Research Endowment.

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volunteers. In all cases, informed consent was obtained. Low-
density BM cells were obtained by separation of theuffy coat cells with Ficoll-Hypaque centrifugation. Nonadherent cells of the Buffy coat were then separated by allowing adherent cells to attach overnight to the bottoms of petri dishes containing Iscove’s modified Dulbecco’s medium (IMDM) and 15% fetal calf serum (FCS; Gibco, Grand Island, NY). The adherent cells were washed several times with Hank’s balanced salt solution (HBSS) plus 15% FCS to ensure removal of nonadherent cells, after which all nonadherent cells were washed and resuspended in IMDM. Cell viability was routinely determined by trypan blue dye exclusion and, in some cases, adherent cells were stained for nonspecific esterases.

Arachidonic acid metabolism. Unseparated human BM and separated BM (adherent and nonadherent populations) were cultured with [1^-14C]-arachidonic acid (Amersham, Arlington Heights, IL, specific activity of 56 mCi/mmol) in the presence and absence of indomethacin (10 μmol/L), SKF-525A (100 μmol/L), or BW-755C (100 μmol/L). The incubation was performed for 30 minutes at 37°C. The reaction was terminated and arachidonic acid metabolites were extracted from both cells and media as described below. In some experiments, cells were homogenized by two cycles of freeze-thaw in distilled water, adjusted to pH 7.4 with 0.2 mol/L potassium phosphate buffer, and incubated with 14C-arachidonic acid in the presence of NADPH (1 mmol/L). The reaction was terminated by acidification to pH 4.0, and arachidonic acid metabolites were extracted with ethyl-acetate. Arachidonic acid metabolites were separated by reverse-phase HPLC and their migration compared with that of authentic standards. HPLC was performed on a C18-Bondapack column (Waters Assoc, Milford, MA) using a linear gradient of 1.25%/min from acetonitrile:water:acetic acid (50:50:0.1) to acetonitrile:acetic acid (100:0.1). Radioactivity was monitored by a flow detector (Radiometric Instrument and Chemical Inc, Tampa, FL). The fractions containing the cytochrome P450-dependent metabolites were pooled, evaporated and resuspended in methanol, and repurified by HPLC. The purified metabolites were stored at -70°C.

Aryl hydrocarbon hydroxylase (AHH) assay. The AHH assay was similar to methods described previously with some modifications. Briefly, BM-adherent cells were scraped and homogenized in cold H2O-saline and incubated for 10 minutes at 37°C in a reaction mixture containing 50 μmol/L benzo(a)pyrene, 1 mmol/L reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 mmol/L reduced nicotinamide adenine dinucleotide (NADH), 1 mmol/L MgCl2, and 0.1 mg bovine serum albumin. Volume was adjusted to 0.6 mL with 0.1 mol/L KH2PO4 buffer, pH 7.8. The reaction was stopped by the addition of 0.5 mL of ice-cold acetone/hexane (3:1). The mixture was vortexed vigorously, and hydroxylated benzo(a)pyrene was extracted into 0.5 mL of 1.0 mol/L of NaOH. The concentration of hydroxylated benzo(a)pyrene in the alkali phase was determined by measuring the fluorescence at 522 nm during excitation at 396 nm using a Perkin Elmer spectrophotometer (Model MP65, Wilton, CT). Duplicate analyses were performed on all samples, and the amount of 3-OH-benzo(a)pyrene generated was determined by referring to a standard curve. The results were obtained after subtracting values for blanks, which were incubated under conditions identical to control incubations but lacked microsomes or cells. Zero-time blanks, prepared with microsomes or cells, showed slight activity; in these incubations, the reaction was stopped immediately after addition of benzo(a)pyrene. Results were expressed as picomoles of 3-OH-benzo(a)pyrene formed per milligram cellular protein per minute of the mean ± SEM of three determinations.

Hematopoietic colony assay. Human BM erythroid colonies (CFU-E, burst-forming unit-erythroid [BFU-E]) were cultured in methylcellulose similar to procedures described previously. Depending on experimental conditions, colonies were grown in the presence of varying amounts of Epo (0.2 to 2.0 U/mL; Toyo, Osaka, Japan) in the absence or presence of BM-derived arachidonic acid metabolites and synthetic 19-HETE and 20-HETE. Synthetic 19- and 20-HETEs were prepared as described previously. Both compounds were labeled at carbon 1 with 14C to a specific activity of 25 cpm/nmol. The compounds were dissolved in ethanol and diluted with phosphate-buffered saline, pH 7.4. The final concentration of ethanol in the culture was 0.05%. The same concentration of ethanol was added to the control cultures. Cultures were then incubated at 37°C in a fully humidified atmosphere of 5% CO2 + 95% air for 7 to 14 days, after which the erythroid colonies were quantitated.

RESULTS

Human BM cells (buffy coat) as a mixed cell population metabolized arachidonic acid via three distinct pathways. These pathways included the classic lipoxygenase and cyclooxygenase pathways as well as the NADPH-dependent cytochrome P450 mixed function oxidase system. The radioactive arachidonic acid metabolites that are formed by BM-adherent cells and recovered in the incubation media (infra vide) were separated on HPLC as seen in Fig 1. These metabolites had similar retention times to authentic standards of cyclooxygenase and lipoxygenase metabolites and included prostaglandins, 5-HETE, 15-HETE, and 5,15 diHETE. In addition, a radioactive peak was seen at 19 minutes. This peak co-migrated with authentic 19- and 20-HETE standards, both of which have proven to be cytochrome P450-dependent metabolites. The presence of the 19-minute migratory peak suggests that the cytochrome P450 system is functional in intact BM cells. We further separated the BM cells into adherent and nonadherent
populations and measured the conversion of arachidonic acid to the cytochrome P450-dependent metabolite(s); the radioactive peak eluted at 19 minutes. The nonadherent cell preparation metabolized arachidonic acid mainly via cyclooxygenase, as shown by the radioactive peaks migrating similarly to prostaglandin authentic standards (Fig 2, upper panel). In contrast, cyclooxygenase activity in the adherent cell preparation was relatively low and two radioactive peaks appeared at 19 and 24 minutes, which comigrated with authentic 20-HETE/19-HETE and 15-HETE, respectively (Fig 2, lower panel). Initially, these experiments were performed by separating the cells from the medium after incubation with arachidonic acid. The free, unbound/nonesterified metabolites and arachidonate were extracted in both preparations, media and cells. The majority of the metabolites, including the 19-minute peak, was found in the incubation media and not inside the cells; 95% of the radioactivity present in the metabolites was in the media. We therefore eliminated the separation step and extracted the metabolites from the whole incubate. These results suggest that the metabolites formed by the cell from exogenous added substrate are released into the medium, indicating that they may be potential humoral mediators. However, to verify that the peak at 19 minutes consisted of cytochrome P450 metabolite or metabolites, cell homogenates from adherent cells were prepared. It is well established that in broken cell preparations the cytochrome P450 pathway can only be expressed when NADPH is added.* As seen in Fig 3, the formation of this peak is evident in the presence of NADPH (Fig 3A) and is not inhibited by BW-755C, a dual inhibitor of cyclooxygenase and lipoxygenase (Fig 3B). In fact, BW-755C caused an increase in the formation of NADPH-dependent metabolites probably by shunting more arachidonic acid through this pathway, ie, the 19-minute peak constituted about 7.2% and 19.2% of total radioactivity recovered in the absence and presence of BW-755C, respectively. Addition of SKF-525A, an inhibitor of cytochrome P450-dependent enzymes, to the reaction system inhibited the formation of the 19-minute peak by 75% (Fig 3C). In the absence of NADPH, the formation of the peak at 19 minutes was very low, about 0.5% of the total recovered radioactivity (Fig 3D), whereas in the presence of NADPH, 7% to 19% of the arachidonic acid was converted to this peak. These results provide substantial evidence that this peak does indeed consist of a cytochrome P450-arachidonate metabolite(s) and that the adherent cells of the BM are a major source of this activity.

Experiments were performed to corroborate the assay results indicating the presence of cytochrome P450-dependent monooxygenase activity within the adherent stromal cells. As shown in Table 1, adherent BM cells incubated with benzo(a)pyrene exhibited an absolute requirement for NADPH or its generating system for benzo(a)pyrene metabolism. Addition of 7-ethoxyresorufin and SKF-525A, known inhibitors of P450 enzymes, inhibited the reaction by 87% and 59%, respectively. The NADPH dependency and the inhibition caused by SKF-525A and 7-ethoxyresorufin indicate that a form of cytochrome P450 is involved in the hydroxylation reaction. The reaction catalyzed by the AHH was linear for 10 minutes.

We next attempted to determine what functional significance the arachidonate product(s) of the cytochrome P450 pathway in BM have on hematopoiesis. Adherent BM cells...
Table 1. AHH Activities of Human Adherent Stromal Cells: Effect of Specific Inhibitors of Monooxygenases

<table>
<thead>
<tr>
<th>Incubation System</th>
<th>Activity (pmol/mg protein/min)</th>
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<tbody>
<tr>
<td>Complete system</td>
<td>6.53 ± 0.50</td>
</tr>
<tr>
<td>- NADPH</td>
<td>1.00 ± 0.30</td>
</tr>
<tr>
<td>+ SKF-525A (100 μmol/L)</td>
<td>2.7 ± 0.47</td>
</tr>
<tr>
<td>+ 7-Ethoxyresorufin (10 nmol/L)</td>
<td>0.8 ± 0.31</td>
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Reaction mixture contained 100 μmol/L potassium phosphate, pH 7.7, 1 μmol/L NADPH, 1 μmol/L MgCl₂, 0.8 mg of adherent stromal cell homogenate, and 50 μmol/L benz(a)pyrene (in 20 μL of acetone). Incubation was in open air in a 12" tube for 10 minutes at 37°C. Results are the mean ± SEM of triplicate determinations.

(5 x 10⁶) were homogenized and incubated with ¹⁴C-arachidonic acid (1 μCi/50 μg cold arachidonic acid) in the presence of NADPH and BW-755C (50 μmol/L) to maximize the amount of the cytochrome P450-arachidonate metabolites formed. The peak at 19 minutes was isolated by HPLC, purified, and its migration on HPLC compared with that of authentic cytochrome P450 metabolites, including 19- and 20-HETE. As seen in Fig 4, the cytochrome P450-arachidonate acid metabolite(s) were formed by human BM-adherent cells that eluted at 19 minutes and comigrated with the cytochrome P450 metabolites, 19- and 20-HETE.

The purification steps yielded about 5 μg of material calculated from the specific activity of arachidonate added. The material was then reconstituted and tested in CFU-E cultures. Figure 5 shows the effect of the isolated 19-minute peak (BM-HETE) on CFU-E growth in the presence of Epo. The results present the mean of five different determinations and demonstrated that addition of estimated amounts of 10⁻¹¹, 10⁻¹⁰, and 10⁻⁹ mol/L of this peak increased the number of colonies of CFU-E by 279%, 360%, and 127%, respectively, ie, 195% ± 38% versus 728% ± 68% for Epo alone and Epo + 10⁻¹⁰ mol/L 19-minute peak, respectively (Fig 5). The vehicle control had no effect on CFU-E growth (data not shown). It has been estimated from the expected molecular weight of NADPH-dependent arachidonic acid metabolites that this 10 ng is equivalent to 10⁻¹⁰ mol/L. Because the HPLC elution profile of the BM-derived cytochrome P450-arachidonate metabolites was identical to both 19- and 20-HETE authentic standards, we tested their effect as well. As seen in Fig 5, both 19- and 20-HETE at 10⁻¹⁰ mol/L potentiate Epo-induced CFU-E growth; however, 20-HETE was more potent.

The erythropoiesis-potentiating effect of 19- and 20-HETE was dose-dependent. As seen in Fig 6, 19- and 20-HETE stimulate Epo-induced CFU-E growth over a wide range of concentrations. 20-HETE was much more potent in stimulating CFU-E growth as compared with the
same concentration of 19-HETE. The maximal stimulation of fourfold over the control (Epo alone) was achieved at $10^{-11}$ mol/L. 20-HETE. 19-HETE had a maximal effect of only a twofold increase on CFU-E growth at $10^{-10}$ mol/L.

Because Epo levels may be critical for the potentiating effect, we next tested different concentrations of Epo on the CFU-E-stimulatory effect of $10^{-10}$ mol/L. 20-HETE. Note in Fig 7 that in the presence of $10^{-10}$ mol/L. 20-HETE there was a shifting to the left of the dose-response curve to Epo. It is also clear from Fig 7 that in the absence of Epo, 20-HETE had no effect on CFU-E growth. In addition, the maximal fourfold stimulation was obtained at 1 U Epo, whereas lower concentrations of Epo resulted in a lesser degree of stimulation (twofold).

**DISCUSSION**

In this study, we have demonstrated that human BM-adherent stromal cells possess a cytochrome P450 "system" that metabolizes arachidonic acid, independent of the classic cyclooxygenase and lipooxygenase pathways, to compounds with powerful erythropoietic-potentiating activity. Through HPLC chromatographic techniques, we were able to demonstrate that these distinct metabolite(s) of arachidonic acid are generated in human BM cells, and significant amounts of these metabolites are recovered in the surrounding media. This microsomal cytochrome P450-dependent system requires NADPH and the generation of metabolite(s) is inhibited by SKF-525A, a cytochrome P450 enzyme inhibitor, which makes it distinct from the other metabolic systems of the arachidonic acid cascade. The inability of indomethacin (a cyclooxygenase inhibitor) and/or BW-755C (a dual inhibitor of cyclooxygenase and lipooxygenase) to inhibit formation of these metabolite(s) further confirms their origin as cytochrome P450-dependent. The depression of cyclooxygenase and lipooxygenase systems with inhibitors resulted in an enhancement of arachidonic acid metabolite formation by the NADPH cytochrome P450 system. These results suggest that either a shunting mechanism exists, or that masking of the cytochrome P450 system/metabolites takes place when other systems ensue. The significance of this remains to be determined. The metabolic product(s) of arachidonic acid formed by the BM-adherent stromal cytochrome P450 system appears to migrate on HPLC in a similar pattern as that of the synthetic compounds 19-HETE and 20-HETE. These compounds have previously been characterized and purified and are both products of the cytochrome P450-arachidonate $\omega$-1 hydroxylases in rat liver and human kidney.22,23

The precise source of metabolizing cells within the BM remains unclear and may involve multiple cell populations. For example, we have demonstrated that the formation of these metabolites was greatest in the adherent cell population of the BM. Macrophage-like cells are included in this population and the presence of nonspecific esterases confirmed their identity in the preparations. We have also shown that cultured macrophages, derived from peripheral human monocytes, are able to metabolize arachidonic acid via a cytochrome P450-arachidonic acid monoxygenase system (unpublished data). In addition, Bedner et al24 and Hatzelmann and Ullrich25 have demonstrated the capacity of unstimulated human polymorphonuclear leukocytes to produce 20-HETE from arachidonic acid. Thus, the myeloid lineages may also be a major source of these cytochrome P450-arachidonic acid metabolites.

The chemical structure of the 19-minute peak formed by the BM cells is not fully identified. Further chemical analysis by mass spectrometry has to be performed to ascertain its chemical structure. Nevertheless, using HPLC coupled with bioassay, we demonstrated that the 19-minute peak produced by the BM has identical retention time to 19- and 20-HETE, and comparable potency as a stimulator of Epo-induced CFU-E growth. These metabolites at a concentration of $10^{-11}$ to $10^{-10}$ mol/L are powerful stimulants of Epo-induced CFU-E growth. It is assumed that a concentration of $10^{-12}$ mol/L of these metabolites is similar to the range of physiologic concentrations and this level can be obtained in vivo under certain conditions. Preliminary experiments demonstrated that 19- and 20-HETE also stimulated BFU-E growth, but to a lesser extent; 50% to 70% stimulation of Epo-induced BFU-E at $10^{-10}$ mol/L of 20-HETE and 19-HETE (unpublished data).

The present study also shows that adherent stromal cells contain a low, but readily detectable, level of other cytochrome P450 monoxygenase systems, as assessed by the metabolism of benzo(a)pyrene. Previous reports have shown the presence and inducibility of AHH in human monocytes and lymphocytes.26 Our results (Fig 5 and Table 1) indicate that the level of cytochrome P450 activities in stromal adherent cells was comparable with that previously measured in the outer medulla of the rabbit kidney,27 human polymorphonuclear leukocytes,24 and human lymphocyte culture.27 Furthermore, these experiments demonstrate that cytochrome P450-arachidonic acid metabolites are released from adherent cells and are present in the cellular incubation mixture. These metabolites may play important roles in hematopoiesis and, in this respect, they could act as paracrine regulators of erythroid progenitor cell growth.

![Fig 7. Dose response of Epo-induced CFU-E growth in the absence (■) and presence (●) of 20-HETE (10^{-10} mol/L). The experimental conditions are the same as in Fig 5.](image-url)
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