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FORMATION AND PROLIFERATIVE EFFECTS OF LIPOXINS IN HUMAN BONE MARROW

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Lipoxins A_4 and B_4 together with the *all-trans* lipoxin (LX) isomers were produced by normal human bone marrow cell suspensions after incubation with ionophore A23187. Both LXA_4 and LXB_4 enhanced the growth of myeloid progenitor cells in semisolid agar in the presence of sub-optimal concentrations of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). Lipoxin A_4 at 10^{-10} M stimulated the colony formation in 13 out of 15 tested human bone marrows with a mean (± SEM) increase of 47 ± 11 % (p=0.001). A similar stimulatory effect was observed after addition of LXB_4 (10^{-10} M). The monohydroxyeicosatetraenoic acids 5-, 12- and 15-HETE did not affect colony growth. In addition, LXA_4 (10^{-8} M) efficiently counteracted the increased colony formation induced by leukotriene C_4 (10^{-10} M), suggesting an antagonistic relationship between these lipoxygenase products. The results support a role for lipoxins in the regulation of human myelopoiesis.

Interaction between the 5- and 15-lipoxygenases can lead to the production of lipoxins (1-3). Recent data also support an important role for the human platelet 12-lipoxygenase in lipoxin formation (4-6). Thus, platelets metabolize granulocyte-derived leukotriene (LT) A_4 to the tetraene epoxide 15-OH-LTA_4, which is further transformed to LXA_4 and LXB_4 (6). Although the biological significance of the lipoxins is not fully elucidated, these compounds have been reported to possess biological activies such as bronchoconstriction, vasodilation, stimulation of protein kinase C and inhibition of NK cells (1). In addition, recent findings indicate actions antagonizing the pro-inflammatory effects of leukotrienes, possibly through competition at common receptors (7-10).

We have recently demonstrated, that platelets from patients with chronic myelogenous leukemia (CML) and other myeloproliferative disorders have a deficient capacity to synthesize lipoxins (11). This defect was particularly pronounced in blastic crisis of CML. Furthermore, peripheral leukocytes from CML patients possess an increased capacity to produce LTC_4 (12). This is of interest, since leukotrienes may act as a stimulators of the myelopoiesis (13-15, Stenke et al, to be published). Although the pathophysiological consequences of these findings are unclear, it may be speculated whether altered formation of lipoxygenase products can contribute to the in vivo regulation of human hematopoiesis. In the present paper we have therefore investigated the capacity of

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human bone marrow to produce lipoxins and analyzed the modulatory action of these compounds on the growth of human myeloid stem cells.

MATERIALS AND METHODS

Reagents

Ficoll-Hypaque (specific gravity 1.078) was manufactured by Pharmacia Fine Chemicals (Uppsala, Sweden). Recombinant human GM-CSF, derived from *E. coli* after hybridization, was purchased from Amersham International (Amersham, UK). Lipoxin A_4 ((5S,6R,15S)-5,6,15-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid) and lipoxin B_4 ((5S,14R,15S)-5,14,15-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid) were obtained from Cayman Chemical Co. (Ann Arbor, MI), while 6(S)-LXA_4 and the *all-trans* isomers of LXA_4 and LXB_4 were kindly provided by Dr K. C. Nicolaou (Univ. of Penn., Phil., PA). Synthetic 5-, 12- and 15-HETE were from Biomol Research Laboratories (Plymouth Meeting, PA). Leukotriene C_4 was a kind gift from Dr J. P. Lellouche (CEN Saclay, Gif-sur-Yvette Cedex, France). Ionophore A23187 was purchased from Calbiochem-Behring (La Jolla, CA).

Cell preparations

Human bone marrow cells from healthy volunteers were collected into heparinized tubes (50 units/ml) by needle aspiration from the iliac crest. All individuals were without medication. Informed consent was obtained from the volunteers and the project was approved by the Ethical Committee of Karolinska Institutet. For investigation of lipoxin synthesis, the bone marrow samples were depleted of erythrocytes by centrifugation (900 x *g*, 15 min) and incubation in hypotonic ammonium chloride. The remaining cells were resuspended in phosphate buffered saline (PBS, Dulbecco's formulation containing 0.9 mM calcium, pH 7.4; Gibco, Paisley, Scotland). For studies on proliferation, the bone marrows were subjected to density centrifugation on a Ficoll-Hypaque gradient to obtain mononuclear cell fractions. After centrifugation at 400 x *g* for 30 min the interphase cells were collected, washed three times and resuspended in PBS. The platelet content in erythrocyte-depleted bone marrow samples was estimated by phase contrast microscopy and found to be 0.5 - 2 platelets per nucleated bone marrow cell. Staining of Ficoll separated bone marrow samples with Türk's solution demonstrated a cell population with > 97% mononuclear cells. The cell viability was > 95% as judged by the trypan blue exclusion test.

Incubation procedure and lipoxin detection

Erythrocyte-depleted bone marrow cell suspensions (1.0 ml containing 10^7 nucleated cells) were equilibrated for 5 min at 37° C and incubated with ionophore A23187 (1 μM) for 30 min. Incubations were terminated by the addition of 5 vol of ethanol. After centrifugation and evaporation, the material was dissolved in 220 μl HPLC mobile phase and subjected to reversed-phase (RP) HPLC using an Ultrasphere-ODS column (4.6 x 150 mm; Beckman, San Ramon, CA) eluted with acetonitrile/methanol/water/acetic acid (24/15/60/0.8; apparent pH 5.6) at a flow rate of 1.0 ml/min. For identification and quantitation of the products, a computerized diode array spectrophotometer (Waters 991, Waters Chromatography Division, Milford, MA) was used. Lipoxin detection was performed by measurements of UV-absorbance at 300 nm.

Colony assay

Mononuclear bone marrow cells were resuspended in a mixture of Iscove's medium (Gibco) with 10 % fetal calf serum (Gibco) and 0.3 % agar (Bacto-Agar, Difco Laboratories, Detroit, MI). One ml portions of the cell suspensions, containing 0.2 x 10^6 cells, were placed in Petri dishes on top of one ml feeder layers. The feeders contained 0.5 % agar in Iscove´s medium and recombinant human GM-CSF, a glycoprotein necessary for the growth and maturation of committed myeloid progenitor cells (16). Various concentrations of lipoxins, HETEs and LTC_4 were added to the cell layers during plating. Stock solutions of the lipoxygenase products were prepared in ethanol and diluted to a final ethanol concentration of 0.05 %. At this concentration ethanol did not affect colony growth. The dishes were incubated at 37° C in an automatically regulated fully humidified atmosphere of 5 % CO_2 in air. Control dishes were made in sets of six, all other dishes in triplicates. All cultured dishes were code numbered. After 10 days of cultivation the growth was scored blindly using an inverted microscope (Zeiss, Germany). Colonies and clusters were defined as containing > 40 cells and 8-40 cells, respectively. Dose-response curves from preliminary experiments indicated that the optimal colony growth was reached with a GM-CSF concentration of 25 units (equivalent to 0.5 ng) per dish. A suboptimal GM-CSF concentration (12.5 units per dish) was then employed in all test dishes. Parallel sets of control dishes were incubated with 12.5 and

25 units of GM-CSF per dish, respectively. Experiments with a mean growth of < 20 colonies per dish in the controls were excluded. The mean values of the relative differences in colony formation between sets of control and test dishes were analyzed using the two-tailed *t*-test of paired samples.

RESULTS

Lipoxin formation in human bone marrow cell suspensions

Ionophore A23187-stimulated suspensions of bone marrow cells produced both lipoxin A_4 and B_4, as well as the *all-trans* isomers of LXA_4 and LXB_4, as determined by RP-HPLC analysis (Fig 1). As judged by on-line UV-spectroscopy, the compounds contained conjugated tetraenes with maximal absorption at 300-302 nm and shoulders at 286-288 and 314-316 nm. Biosynthetic compounds were identified by cochromatography with synthetic standards. Under the conditions used the lipoxin production was in the low picomolar range.

Effect of lipoxins and HETEs on human myeloid progenitor cell growth

In the absence of GM-CSF, no growth of colonies or clusters could be detected in bone marrow cultures with or without various concentrations of lipoxins. In control dishes the optimal concentration of GM-CSF (25 units per dish) induced the formation of 103 ± 15 colonies per dish (mean ± SEM), while 72 ± 9 colonies per dish (n=18, p=0.001) were formed in the presence of the selected suboptimal GM-CSF concentration (12.5 units per dish). The effect of lipoxins in cultures with the suboptimal GM-CSF concentration is displayed in Fig 2. Lipoxin A_4 at 10^{-10} M increased the number of colonies in 13 out of 15 tested bone marrow samples, with a mean increase of 47 ± 11 % (± SEM) as compared to control dishes without LXA_4 (p=0.001). The maximal increase observed with 10^{-10} M LXA_4 was 136 %, with an increment from 88 to 208 colonies per dish. The equivalent concentration of LXB_4 also induced a significant (p=0.018) stimulation of progenitor cell proliferation, increasing the colony growth in 12 out of 15 tested bone marrows, mean increase 44 ± 16 % (maximal individual increase 200 %; from 32 to 96 colonies per dish). Addition of higher or lower concentrations (10^{-8} M and 10^{-12} M) of either LXA_4 or LXB_4 failed to significantly affect the colony formation (Fig 2).

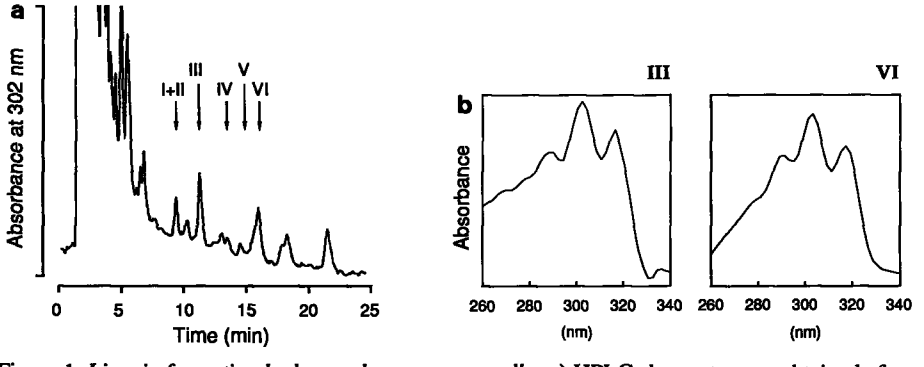

Figure 1. *Lipoxin formation by human bone marrow cells.* a) HPLC chromatogram obtained after incubation of 1.0 ml bone marrow cell suspension, containing 10^7 nucleated cells, with 1 μM A23187 for 30 min at 37° C. Retention times of synthetic standards are indicated by arrows; I+II: *all-trans*-LXB_4; III: LXB_4; IV+V: *all-trans*-LXA_4; VI: LXA_4 b) UV-spectra of material in peak III and peak VI.

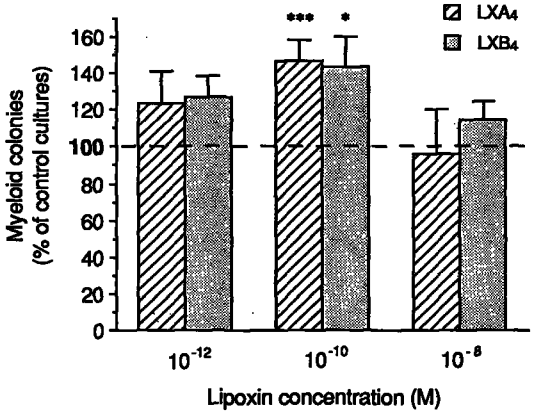

Figure 2. *Effect of lipoxins on human myeloid progenitor cell growth.* The number of colonies at day 10 in GM-CSF stimulated bone marrow cultures co-cultivated with lipoxins (10^{-12} - 10^{-8} M) was compared to that of corresponding control cultures without lipoxins. The bars represent the mean values (± SEM) from experiments with bone marrow cells from 8 - 15 healthy volunteers (LXA_4: 10^{-12} M, n=10; 10^{-10} M, n=15; 10^{-8} M; n=10. LXB_4: 10^{-12} M, n=8; 10^{-10} M, n=15; 10^{-8} M, n=9). Statistical analyses were made against control dishes (paired *t*-test). * $p < 0.05$ *** $p < 0.001$.

In contrast to the lipoxins, the monohydroxy acids 5-, 12- and 15-HETE (10^{-12} M - 10^{-8} M) did not alter the colony formation of bone marrow cells stimulated with suboptimal GM-CSF concentrations (Table I).

Effects of lipoxins on LTC_4-induced stimulation of myeloid progenitor cell growth

Leukotriene C_4 at 10^{-10} M enhanced the colony formation in eight GM-CSF stimulated bone marrow samples with a mean increase of 42 ± 14 % (p=0.023). When LTC_4 was added together with an excess of LXA_4 (10^{-8} M), this stimulation was reduced in all cases and the net colony

Table I

Concentration	% of controls		
	5-HETE	12-HETE	15-HETE
10^{-12} M	90.0 ± 7.0 %	92.5 ± 10.1 %	89.6 ± 11.4 %
10^{-10} M	103.2 ± 8.8 %	113.2 ± 10.1 %	120.1 ± 6.8 %
10^{-8} M	85.8 ± 14.9 %	111.8 ± 12.6 %	88.5 ± 17.8 %

Effect of HETEs on human myeloid progenitor cell growth. The number of colonies at day 10 in GM-CSF stimulated bone marrow cultures co-cultivated with 5-, 12-or 15-HETE (10^{-12} - 10^{-8} M) was compared to that of corresponding cultures without monohydroxy acids. The results represent the mean values (± SEM) from experiments with bone marrow cells from five (12-HETE) or four healthy volunteers.

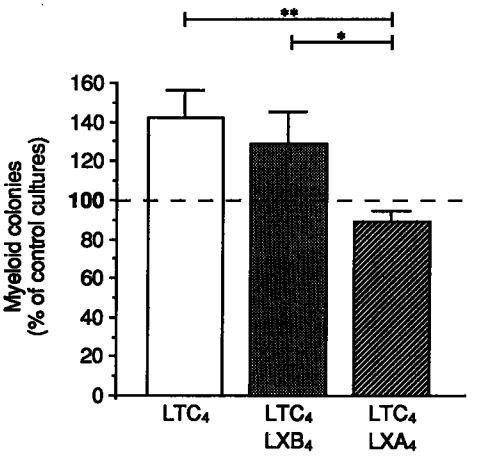

Figure 3. *Effects of lipoxins on leukotriene C_4-induced stimulation of myeloid progenitor cell growth.* Bone marrow cells from 8 healthy donors were incubated in the presence of GM-CSF and leukotriene C_4 (10^{-10} M), with or without lipoxins A_4 or B_4 (10^{-8} M). The bars represent the mean (+SEM) effect of addition of lipoxygenase products on the number of colonies after ten days of cultivation. Statistical analyses were made using paired *t*-test.
* $p < 0.05$ ** $p < 0.01$.

formation returned to levels equivalent to those of the controls (Fig 3). In contrast, LXB_4 (10^{-8} M) did not counteract the stimulatory effect of LTC_4 on the myeloid progenitor cell growth.

DISCUSSION

This paper presents novel data demonstrating the capacity of human bone marrow to synthesize lipoxins and suggesting a modulatory role for the lipoxins in human myelopoiesis. Lipoxins A_4 and B_4 were formed after incubation of bone marrow cell suspensions with the calcium ionophore A23187. The cellular origin of these products is unknown, but since eosinophilic granulocytes transform endogenous arachidonic acid to lipoxins (17, 18), these cells, although constituting a minor part of the bone marrow cell population, may have contributed to the production. It may also be speculated whether less mature myeloid cells were capable of lipoxin formation from endogenous substrate. In addition, transcellular conversion of leukocyte-derived LTA_4 to lipoxins may have occurred (4-6). Thus, the bone marrow contains platelets that may have participated in this formation. Also, reticulocytes of the bone marrow may have transformed LTA_4 to lipoxins, since these cells express 15-lipoxygenase activity (3, 19).

The lipoxins could not induce growth of human bone marrow progenitor cells in a semisolid agar system supplemented only with culture medium and serum. However, highly significant, synergistic effects were observed when low concentrations (10^{-10} M) of LXA_4 or LXB_4 were added to myeloid progenitor cells in the presence of the growth factor GM-CSF. This cytokine was used in a suboptimal concentration, since preliminary experiments indicated less pronounced synergistic effects of lipoxins in the presence of optimal concentrations of GM-CSF (data not shown). The lipoxin-induced effects appeared to be dose dependent resulting in a bell-shaped dose-response curve. Thus, 100-fold higher or lower concentrations of LXA_4 or LXB_4 failed to clearly stimulate the colony growth. In contrast to the lipoxins, the related lipoxygenase products 5-, 12- and 15-

HETE did not influence the stem cell growth, supporting a degree of specificity of the potent effects exerted by the lipoxins. It was recently reported, that 10^{-9} M LXB_4 increased the colony formation from human peripheral mononuclear cells placed in diffusion chambers in the peritoneal cavity of CBA mice during seven days (20). The present data demonstrate, that both lipoxins A_4 and B_4, in concentrations that can possibly be formed locally in the bone marrow, modulate the growth of human myeloid progenitor cells.

Leukotriene C_4 has previously been reported to potentiate the proliferation of bone marrow derived stem cells in vitro (14, 15). In the present investigation this LTC_4-induced stimulation could be reversed by an excess of LXA_4, but not by LXB_4. The lipoxins alone exerted no potentiating or inhibiting activities at this concentration. These results indicate an antagonistic relationship between LTC_4 and LXA_4. In other systems it has indeed been demonstrated that LXA_4 antagonizes the cysteinyl-containing leukotrienes, possibly by sharing the same receptor. Thus, LTD_4 receptor antagonists reduced LXA_4 mediated bronchoconstriction (21). Furthermore, LXA_4 inhibited LTD_4 binding to its receptor (7) and attenuated LTD_4-induced granulocyte adhesion to mesangial cells (10). Lipoxin A_4 also reversed LTD_4-mediated decrease of glomerular ultrafiltration rate in the rat together with changes in inositol triphosphate levels in cultured mesangial cells (7).

The possible mechanisms behind the described lipoxin effects on myeloid progenitor cell growth are at present unknown. Since the stimulation was significant at suboptimal concentrations of GM-CSF, it is conceivable that LXA_4 and LXB_4 may act through enhancement of endogenous cytokine production. However, it should be noted, that lipoxins totally failed to induce any colony growth in the absence of GM-CSF. Molecular biological techniques may facilitate the elucidation of possible lipoxin-induced expression of GM-CSF or other cytokines.

In summary, the present results demonstrate lipoxin synthesis in the bone marrow and link this finding to a potent biological effect in the same tissue. The lipoxin-induced modulation of myeloid stem cell growth, together with the observed capacity to produce these compounds, may indicate a regulatory role for the lipoxins in the human bone marrow. However, the precise nature of this regulation, needs to be further clarified, since e.g. LXA_4 induced both synergistic (with GM-CSF) and antagonistic (with LTC_4) effects on progenitor cell growth.

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