Arachidonic acid and freshly isolated human bone marrow mononuclear cells

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Arachidonic acid (AA), a fatty acid found in the human bone marrow plasma, is the precursor of eicosanoids that modulate bone marrow haematopoiesis. To further our understanding of the role of AA in the bone marrow physiology, we have assessed its incorporation in human bone marrow mononuclear cells. Gas chromatography analysis indicates the presence of AA in their fatty acid composition. In bone marrow mononuclear cells, [3H]-AA is incorporated into triglycerides and is later delivered into phospholipids, a result not observed with blood mononuclear cells. Prelabelling-chase experiments indicate a trafficking of labelled AA from phosphatidylcholine to phosphatidylethanolamine. Stimulation of prelabelled bone marrow mononuclear cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) results in the release of a part of the incorporated labelled AA. Finally, exogenous AA (up to 1 μM) has no significant effect on cell growth. In conclusion, human bone marrow mononuclear cells participate to the control of marrow AA concentrations by incorporating AA into phospholipids and triglycerides. In turn, bone marrow mononuclear cells can release AA in response to the potent haematopoietic growth factor GM-CSF.

Key words: Arachidonic acid, GM-CSF, Mononuclear marrow cells, Proliferation

Introduction

Arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, is converted to prostaglandins through the cyclooxygenase pathway or to hydroxyeicosatetraenoic acid (HETE) and to leukotrienes through the lipoxygenase pathway.[1] Several AA metabolites modulate human haematopoiesis by acting on the growth of human bone marrow erythroid and myeloid progenitors,[2-5] and on the cytokine production by human bone marrow stromal cells.[6] The role of AA in the bone marrow physiology is poorly documented. Recently we have reported the presence of AA in the human bone marrow plasma and its capture by cultured human bone marrow stromal cells.[7] To further our understanding of the role of AA in the bone marrow, the uptake and the distribution of AA among triglycerides (TG) and phospholipids (PL) in human bone marrow mononuclear cells were compared with those in blood mononuclear cells and erythrocytes. We have also investigated the role of AA on the proliferation of human bone marrow mononuclear cells and their capacity to release AA in response to granulocyte-macrophage colony stimulating factor (GM-CSF), a potent haematopoietic growth factor.

Material and Methods

Reagents

AA (Tebu, Le Perray en Yvelines, France) was dissolved (0.1 M) in ethanol stored at –80°C, and appropriately diluted in 2% human serum albumin (HSA) from Bio Merieux (France) immediately before use. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), neutral lipid (NL), bovine serum albumin (BSA) and standard solutions of monoglycerides (MG), diglycerides (DG), TG and PL were from Sigma (Saint Quentin Fallavier, France). Hanks's balanced salts solution (HBSS), RPMI 1640, penicillin, streptomycin and fetal calf serum (FCS) were from Gibco (Cergy Pontoise, France). [3H]-thymidine and [3H]-AA were from Amersham (Les Ulis, France). GM-CSF was from R&D systems (Oxon, UK).

Human bone marrow cells from patients undergoing a myelogram as part of a diagnostic procedure were harvested by aspiration into heparinized tubes (Vacutainer system, Becton Dickinson, Meylan, France). When samples were considered as normal by morphological analysis of the May Grunwald Giemsa stained smears, bone marrow mononuclear cells were isolated by separation on a Ficoll gradient (400 $\times$ **g**,

20 min) and washed twice with HBSS (400 × **g**, 10 min) before use. For preparation of blood mononuclear cells, human blood was drawn from healthy volunteers using heparin as anticoagulant and prepared as bone marrow cells. For preparation of erythrocytes, blood was recovered using heparin as anticoagulant. The buffy coat was aspirated and discarded. The red cells were washed three times with HBSS before use.

Fatty acid analysis of human bone marrow mononuclear cells

Cell lipids were extracted from freshly isolated mononuclear bone marrow cells according to Floch *et al.*[8] Fatty acids were then methyl transesterified and the resulting fatty acid methyl esters were separated by gas chromatography.[9] Standards were run to identify the compounds quantified by gas chromatography.

Incorporation of [³H]-AA in cells

The incorporation of [³H]-AA was performed with 5 × 10⁶ human bone marrow mononuclear cells, 5 × 10⁶ blood mononuclear cells and 5 × 10⁷ blood erythrocytes maintained in 1 ml of RPMI 1640 medium without FCS. [³H]-AA (0.1 µCi; 210 Ci/mmol) was added into the medium for various periods of time. At the end of the experiments, cells were harvested by centrifugation (800 × **g**, 10 min) and then stored at –20°C until used. For AA remodelling studies, mononuclear bone marrow cells were labelled with [³H]-AA for 0.5 h, washed with HBSS, and incubated in 1 ml of RPMI 1640 without FCS for 2, 5 and 22 h or used immediately.

Lipid extraction

Cells were mixed with 1 ml of 0.1% sodium dodecyl sulphate in water, and incubated for 30 min at 56°C with 3 ml of chloroform/methanol (2:1, v/v). The chloroformic extract was washed with 5 ml of 0.1 M KCl/methanol/chloroform (96:92:6, v/v/v), and was evaporated.[10] The dry extract was recovered in 150 µl of chloroform and applied to a thin layer chromatography (TLC) plate (Silica gel 60 (20 × 20 cm, 0.25 mm), Merck) and submitted to TLC. The plate was developed in the mixture of diethyl ether/hexane/acetic acid (70:30:1, v/v/v). Each lane was divided in areas of 0.5 cm length which were scraped into vials and radioactivity was measured on a Packard liquid scintillation counter. Solutions of MG, DG, TG, PL and AA were used as standards and visualized with iodine vapour.

Analysis of labelled PL

For the separation of the various species of cellular PL, the corresponding areas were scraped from the TLC plates and were extracted with chloroform/methanol (2:1, v/v). Samples were then rechromatographed using a solvent system of chloroform/methanol/acetic acid/water (50:30:8:4, v/v/v/v).[10] Each lane was divided in areas of 0.5 cm length and processed as above. Solutions of PC, PS, PI, and PE were used as standards and visualized with iodine vapour. In this chromatography, PI and PS migrated to the same area.

To ensure that the labelled compound in cellular PL from freshly-isolated marrow mononuclear cells was authentic AA, labelled PL were submitted to base hydrolysis.[11] The labelled PL were resuspended in 1 ml of 2 M KOH in ethanol/water (3:1, v/v). After 40 min at 60°C, 1 ml of water and 1 ml of 6 N HCl were added to the mixture to acidify the phase (pH 3). Labelled compounds were extracted with chloroform/methanol (2:1, v/v) and rechromatographed using a solvent system of diethyl ether/hexane/acetic acid (70:30:1, v/v/v). The amount of radioactivity migrating with PL and free AA was determined by liquid scintillation counting. In another set of experiments labelled PL were hydrolysed with PLA₂ from bovine pancreas (Sigma). Briefly, labelled PL samples were resuspended in 1 ml of diethyl ether/methanol (95:5, v/v) containing 50 µl of Tris buffer (0.05 M), CaCl₂ (25 mM), EDTA (1 mM), and 1 mg of phospholipase. The reaction mixture was incubated at 37°C for 2 h with continuous shaking. After evaporation of solvent, samples were rechromatographed as for base hydrolysis.

Proliferation studies

Freshly-isolated bone marrow mononuclear cells (1 × 10⁵ per well) were cultured in 96-well microtitre plates in 100 µl of RPMI 1640 with 10% FCS in the presence of AA (from 1 µM to 0.1 nM) or the appropriate vehicle (10 µl 2% HSA). After 64 h of incubation, cultures were pulsed for 8 h with 1 µCi/ml [³H]thymidine and cells were harvested using a Skatron cell harvester. Each experiment was carried out as sixplicates. In separate sets of experiments, cells (in triplicate samples) were harvested and counted by using a haemocytometer. Data were compared using the Student's *t*-test for paired variables.

Release of AA from [³H]-labelled bone marrow mononuclear cells

Freshly-isolated human bone marrow mononuclear cells were labelled for 0.5 h with [³H]-AA as reported above. After washing, labelled cells (1 × 10⁶) were

suspended in 1 ml of RPMI 1640 containing 0.25% fatty acid-free BSA, 1 mM Ca^{2+} and 1 mM Mg^{2+}. Cells were then incubated at 37°C in the absence or presence of GM-CSF (100 ng/ml). After various periods of time (from 1 to 60 min) the mixture was centrifuged (5 min, 2000 × **g**) and the [³H]-supernatant fluid was removed into a separate vial. Radioactivity in the packed labelled cells and in the radioactive supernatant fluid were then measured on a Packard liquid scintillation counter.

Results

Fatty acid composition of human bone marrow mononuclear cells

As shown in Table 1, AA is present in freshly isolated human bone marrow mononuclear cells.

Incorporation of [³H]-AA in cellular lipid species

After 5 h of incubation with [³H]-AA, freshly-isolated bone marrow mononuclear cells, blood mononuclear cells and erythrocytes had incorporated 52%, 49% and 41% of the initially added radioactivity, respectively (mean of three experiments). Lipids were extracted from labelled cells and separated into classes by TLC. The distribution of label in cell lipids as a function of time is shown in Fig. 1. A trafficking of labelled AA from TG to PL was observed with freshly-isolated human bone marrow mononuclear cells (Fig. 1A) but not with blood mononuclear cells and erythrocytes (Fig. 1B and 1C, respectively). For mononuclear bone marrow cells at 24 h, 67 ± 8% of the label was recovered in PL while 30 ± 7% was recovered in TG. Less than 3% of label was recovered in DG or as free AA. Labelled MG were not detected.

Incorporation of [³H]-AA in cellular PL species

We next investigated the distribution of [³H]-AA into PL species using mononuclear bone marrow cells

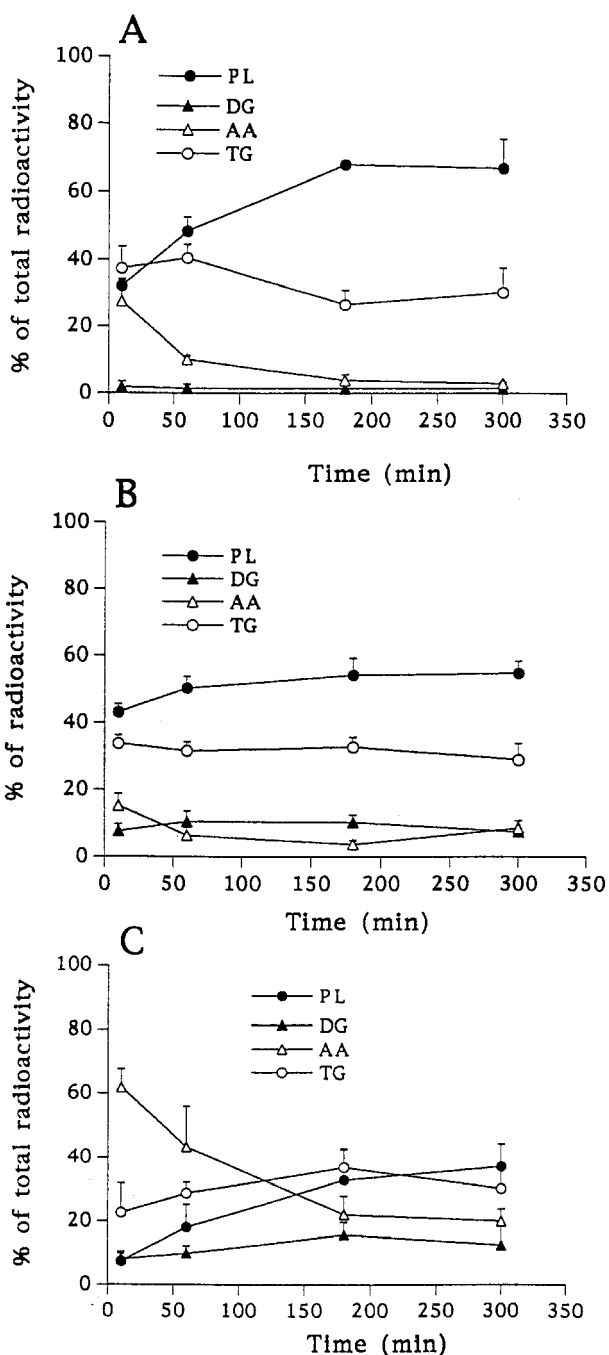

FIG.1. Time-course of [³H]-AA incorporation into bone marrow mononuclear cells (A), blood mononuclear cells (B) and erythrocytes (C). Cells were incubated with [³H]-AA for 10 min, 1, 5, or 24 h. Lipids were extracted and separated as described in Methods. Results are expressed as the percentage of total radioactivity found in cells. Mean ± SEM of three independent experiments in duplicate. PL: phospholipids; DG: diglycerides; TG: triglycerides; AA: arachidonic acid.

Table 1. Fatty acid composition of human bone marrow mononuclear cells. Data (mean ± SEM of four independent samples) are reported as the percentage of fatty acid species

Major fatty acids	Percentage
C14:0	1.6 ± 0.2
C16:0	19.4 ± 1.0
C16:1	3.0 ± 0.5
C18:0	14.4 ± 6.3
C18:1	28.3 ± 4.4
C18:2	12.3 ± 2.7
C20:4	3.4 ± 0.7
Others*	17.4 ± 5.1

*Others: includes the sum of all other fatty acids (not shown) that were <2% of total fatty acids. C20:4 is arachidonic acid.

labelled for 0.5 h. The percentage (mean of three experiments) of incorporated AA into PE (43.7 ± 4.3%) was similar to that for PC (42.0 ± 3.6%) and higher than for PI + PS (14.1 ± 1.6%). We then investigated the putative exchange of labelled AA between PL species. Freshly-isolated bone marrow
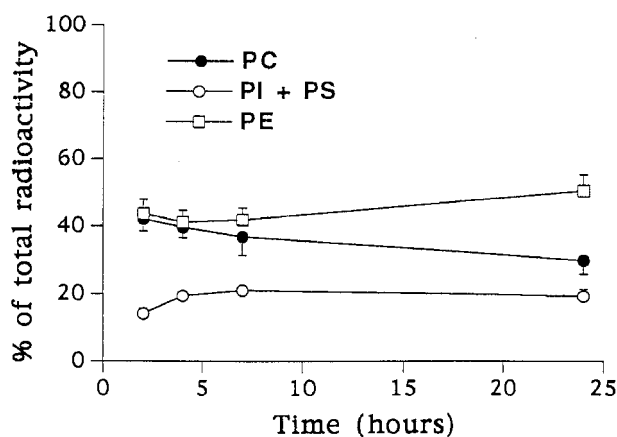
FIG. 2. Trafficking of [³H]-AA into PL species of bone marrow mononuclear cells. Cells were labelled for 0.5 h with [³H]-AA. Medium was removed and cells were incubated with medium free of [³H]-AA for 2, 5 or 22 h. Results are expressed as the percentage of total radioactivity found in PL species. Mean ± SEM of three independent experiments in duplicate. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI + PS: phosphatidylinositol + phosphatidylserine (comigration).
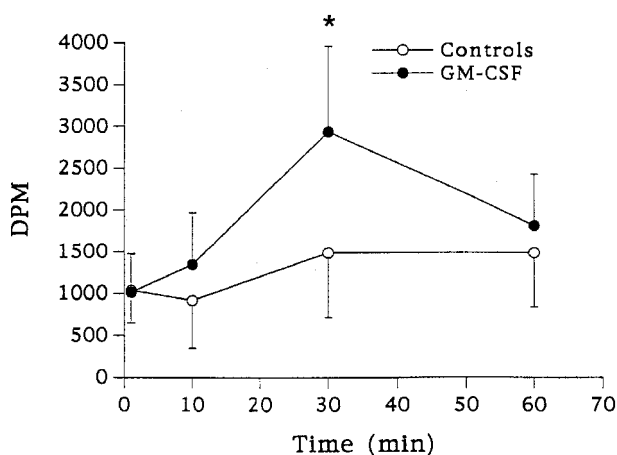

FIG. 3. Release of [³H]-AA by bone marrow mononuclear cells. Labelled cells were stimulated with GM-CSF (100 ng/ml) at 37°C for various periods of time. Radioactivity in cell supernatants was measured on a liquid scintillation counter. Mean ± SEM of three independent experiments. *$P < 0.05$ compared with controls (Mann-Whitney U-test).

mononuclear cells labelled for 0.5 h were grown in culture medium free of [³H]-AA for 2, 5 or 22 h. Under these experimental conditions, a trafficking of [³H]-AA from PC to PE was documented (Fig. 2).

Base hydrolysis of labelled PL from bone marrow mononuclear cells resulted in the loss of 99% (mean of three experiments) of radioactivity, which was recovered as free fatty acid. Similarly, treatment of labelled PL with PLA_2 from bovine pancreas lead to the loss of 80% (mean of two experiments) of label.

Release of [³H]-AA by bone marrow mononuclear cells

Prelabelled human bone marrow mononuclear cells were challenged with GM-CSF (100 ng/ml) for various periods of time. As reported in Fig. 3, GM-CSF stimulated in a time-dependent manner [³H]-AA release by human bone marrow mononuclear cells. Their amounts of [³H]-AA released in response to 1 h of stimulation with GM-CSF represented 11.4 ± 2.9% (mean ± SEM of three independent experiments) of their total [³H]-AA content.

Effect of AA on the growth of human bone marrow mononuclear cells

As reported in Table 2, the addition of AA (1 µM to 1 nM) had no significant ($P > 0.1$) effect on the [³H]-thymidine incorporation by freshly-isolated human bone marrow mononuclear cells cultured with 10% FCS in medium. No significant ($P = 0.5$, Mann-Whitney U-test, three experiments) effect of AA (1 µM) was found after 3 days of growth on the number of viable mononuclear marrow cells (87.4 ±

10.4×10^3 cells) compared with controls (88.5 ± 13.7 $\times 10^3$ cells).

Discussion

AA metabolism plays a pivotal role in many cellular functions,[1] and AA metabolites act on human marrow haematopoiesis *in vitro*.[2-5] Recently we have reported the presence of AA in the human bone marrow plasma.[7] To further our understanding of the role of AA in the human bone marrow, we have investigated the capacity of human bone marrow mononuclear cells to capture and to release AA as well as the putative role of AA in their growth.

Using gas chromatography analysis, we found that AA is present in freshly-isolated human bone marrow mononuclear cells. Similarly to what was observed with human bone marrow stromal cell cultures,[7] experiments with [³H]-AA indicate that freshly-isolated bone marrow mononuclear cells incorporate AA into PL and TG. Time-course experiments reveal a transfer of AA from TG to PL. As already shown in several inflammatory cell types, the incorporation of AA into TG is important to capture fatty acids for PL biosynthesis but also to regulate AA metabolism.[12,13] In contrast to bone marrow mononuclear cells, no transfer of AA from TG to PL is observed with blood mononuclear cells. As previously reported for various kinds of mammalian erythrocytes,[14,15] human erythrocytes incorporate exogenous AA into PL and TG. Their lack of transfer of AA from TG to PL might be related to their inability of *de novo* PL synthesis.[12]

The analysis of PL species of human bone marrow mononuclear cells indicates a preferential incorporation of AA into PE and PC. Similarly to what was observed in several inflammatory cell types,[12,16] prelabelling-chase experiments indicate a trafficking

of labelled AA from PC to PE. At this time the meaning of this traffic is unclear. PLA$_2$-treatment and base hydrolysis experiments confirm that [^{3}H]-AA is incorporated unmodified in mononuclear bone marrow PL and is linked by an ester bond. Stimulation of prelabelled bone marrow mononuclear cells with GM-CSF results in the hydrolysis of a part of the incorporated labelled AA. This effect might be mediated through the activation of a cellular phospholipase A$_2$ (PLA$_2$) activity. Strenghtening this hypothesis GM-CSF generates PLA$_2$ activity with the release of free fatty acids from neutrophil plasma membranes.[17]

Exogenous

AA does not change the growth of human bone marrow mononuclear cells assessed by [^{3}H]-thymidine incorporation into DNA and cell counts. In similar experimental conditions several lipoxygenase metabolites of AA have no effect on their growth.[18] As a positive control of cell stimulation we may mention that in similar experimental conditions, the lipid mediator platelet-activating factor (PAF) stimulates their [^{3}H]-thymidine incorporation.[19]

In conclusion human bone marrow mononuclear cells might participate to the control of marrow AA concentrations by incorporating AA into their PL and TG. In turn, the potent haematopoietic growth factor GM-CSF stimulates their AA release, a result of importance considering the role of eicosanoids during human marrow haematopoiesis.[2]

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