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Incorporation and effect of arachidonic acid on the growth of human myeloma cell lines

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The objectives of this work are to investigate the incorporation of arachidonic acid (AA) in the human myeloma cell lines OPM2, U266 and IM9, and to assess the effect of AA and lipoxygenase products of AA on their growth. The kinetics of acylation of [^{3}H]AA indicates that myeloma cells incorporate AA into their membrane phospholipids and triglycerides. PLA_2-treatment and base hydrolysis experiments confirm that [^{3}H]AA is incorporated unmodified in U266, IM9 and OPM2 phospholipids, and is linked by an ester bond. Prelabeling-chase experiments indicate no trafficking of labeled AA among the various phospholipid species. Addition of AA and lipoxygenase products of AA (leukotriene B_4 and C_4, lipoxin A_4 and B_4, 12- and 15-hydroxyeicosatetraenoic acid) have no effect on U266, IM9 and OPM2 proliferation assessed by [^{3}H]thymidine incorporation into DNA. In conclusion, while human myeloma cells readily incorporate AA in their membrane phospholipids and triglycerides, AA and lipoxygenase products are not important modulators of their proliferation.

Key words: Arachidonic acid, Myeloma cells, Lipoxygenase metabolites, Proliferation

Introduction

The lipoxygenase pathway converts arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, in hydroxyeicosatetraenoic acid (HETE), lipoxins (LX) and leukotrienes (LT).[1] Lipoxygenase inhibitors enhance the proliferation of human B cells *in vitro*,[2] while they have antiproliferative effects on malignant hematopoietic cell lines,[3,4] thus suggesting a role of endogenous lipoxygenase metabolites of AA during cell proliferation. While exogenous lipoxygenase products modulate cell proliferation in several cell types,[5–7] their effect (if any) on the growth of human B cell lines are not documented. In this study we have investigated the incorporation of AA in three human myeloma cell lines (i.e. IM9, U266 and OPM2) and the effect of AA and lipoxygenase metabolites, such as LTB_4, LTC_4, LXA_4, LXB_4, 12-HETE and 15-HETE, on their proliferation.

Materials and Methods

Incorporation of [^{3}H]AA in myeloma cells

U266, IM9 and OPM2 were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in suspension in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Gibco, Cergy Pontoise, France), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in 5% CO_2 in air. Before use, cells were washed twice in Hanks' balanced salts solution (HBSS) and numerated. The incorporation of [^{3}H]AA (Amersham, Les Ulis, France) was performed with cells (3×10^6) in 1 ml of RPMI 1640 medium without FCS. [^{3}H]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 ($800\times g$, 10 min) and then stored at –20°C until use. For AA remodeling studies, cells were labeled with [^{3}H]AA for 30 min, washed with HBSS, and incubated in 1 ml of RPMI 1640 without FCS for 7 h or used immediately. For these experiments cells were stored as above.

Lipid extraction

Cells were mixed with 1 ml of 0.1% sodium dodecyl sulfate 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.[8] 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).[9] Each lane was divided into areas of 0.5 cm in length which were scraped into vials, and radioactivity was measured on a Packard liquid scintillation counter. Solutions of monoglycerides

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(MG), diglycerides (DG), triglycerides (TG), phospholipids (PL) and AA (Sigma, Saint Quentin Fallavier, France) were used as standards and visualized with iodine vapor.

Analysis of labeled 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).[8] Each lane was divided into areas of 0.5 cm in length and processed as above. Solutions of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) (Sigma) were used as standards and visualized with iodine vapor. In this chromatography, PI and PS migrated to the same area.

Base hydrolysis and PLA2 treatment of labeled PL

In a first set of experiments, the labeled PL were resuspended in 1 ml of 2 M KOH in ethanol/water (3:1, v/v) as previously reported.[9] 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). Labeled 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, labeled PL were hydrolysed with PLA_2 from bovine pancreas (Sigma). Briefly, labeled PL samples were resuspended in 1 ml of diethyl ether/methanol (95:5, v/v) containing 50 µl of Tris buffer (0.05 M), $CaCl_2$ (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

U266, IM9 and OPM2 cells (5×10^5 per well) were cultured in 96-well microtiter plates in 100 µl of RPMI 1640 with 10% FCS in the presence of AA, LTB_4, LTC_4, LXA_4, LXB_4, 12-HETE and 15-HETE (1 µM) or the appropriate vehicle (10 µl of 2% human serum albumin).[10] After 64 h of incubation, cultures were pulsed for 8 h with 1 µCi/ml of [^{3}H]thymidine and cells were harvested using a Skatron cell harvester. Each experiment was carried out on n=6 samples. Data were compared by Mann–Whitney U-test. A $P<0.05$ was considered significant.

Results and Discussion

As reported in Fig. 1, OPM2, U266 and IM9 incorporate [^{3}H]AA into their membrane lipids. The distribution of label in cell lipids as a function of time indicates that the majority of label is found with TG

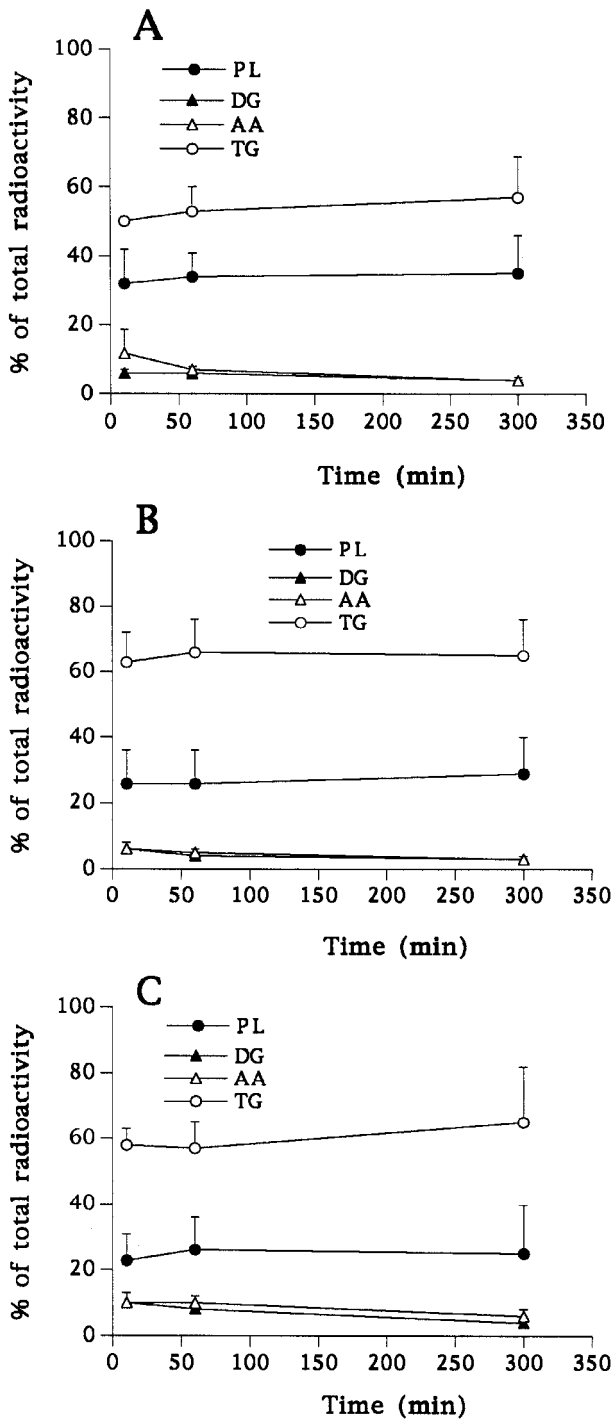

FIG. 1. Time-course of [^{3}H]AA incorporation into U266 (A), OPM2 (B) and IM9 (C) cell lipids. Cells (3×10^6) were incubated with [^{3}H]AA for 10, 60 and 300 min. Cell lipids were extracted and separated as described in Materials and Methods. Results are expressed as the percentage of total radioactivity found in cells: mean±S.E.M. of three independent experiments in duplicate. PL, phospholipids; DG, diglycerides; TG, triglycerides; AA, arachidonic acid.

and PL. Less than 10% of label is recovered in DG or as free AA, and labeled MG are not detected. A transfer of AA from TG to PL has been reported in several human cell types.[11] This trafficking is not found in U266, IM9 and OPM2 cells. Authors have suggested that the incorporation of [^{3}H]AA into the different cellular lipid species does not exactly reflect the distribution of the arachidonate mass in them, since the exogenous [^{3}H]AA might be incorporated into the less abundant AA-containing lipid species.[12] In our experimental conditions the addition of unlabeled AA (0.1 μM) into culture medium has no significant effect on the incorporation and distribution of [^{3}H]AA in PL and TG of U266, IM9 and OPM2 cells (data not shown). Finally the base hydrolysis of labeled PL from myeloma cells results in the loss of 98% (mean of three experiments) of radioactivity, which was recovered as free fatty acid. Similarly, treatment of labeled PL with PLA_2 from bovine pancreas leads to the loss of 90% (mean of three experiments) of label. Thus, base hydrolysis and PLA_2-treatment experiments indicate that [^{3}H]AA is incorporated unmodified in myeloma cell PL and is linked by an ester bond.

The distribution of [^{3}H]AA into PL species using myeloma cells labeled for 30 min is reported in Table 1. PC and PE are the major PL species in OPM2, IM9 and U266 cells. Prelabeling-chase experiments have documented a trafficking of labeled AA from PC to PE in several inflammatory cell types.[13] To investigate the putative exchange of labeled AA between PL species, myeloma cells labeled for 30 min were washed and grown in culture medium free of [^{3}H]AA for 7 h. Under these experimental conditions no trafficking of [^{3}H]AA from PC to PE is documented for OPM2, U266 and IM9 cells (data not shown). Studies report that lipoxygenase and cyclooxygenase products of AA might derive from different PL species in some inflammatory cell types.[12] Thus, two sources of AA are released during immunological activation of mast cells.[14] PC provides AA for LTs biosynthesis, while PE provides the released AA that might be used for the production of cyclooxygenase products. Clearly the role of the different PL species on the production of lipoxygenase and cyclooxygenase metabolites of AA by human myeloma cells deserve to be investigated.

Table 1. Incorporation of [^{3}H]AA into PL species of OPM2, U266 and IM9 cells

	PC (%)	PI + PS (%)	PE (%)
OPM2	59 ± 3	17 ± 1	24 ± 4
IM9	35 ± 3	27 ± 1	38 ± 5
U266	53 ± 5	18 ± 1	29 ± 5

Cells (3×10^6) were labeled for 0.5 h with [^{3}H]AA. Results are expressed as the percentage of total radioactivity found in PL species: mean ± S.E.M. of three independent experiments in duplicate. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI+PS, phosphatidylinositol+phosphatidylserine (comigration).

Table 2. Effects of AA and lipoxygenase metabolites on [^{3}H]thymidine incorporation of U266, OPM2 and IM9 cells

	U266	OPM2	IM9
Controls	16492 ± 1984	288474 ± 40225	5784 ± 510
AA	13897 ± 1111	268889 ± 29266	5627 ± 822
LTB_4	15836 ± 1731	264995 ± 26473	6052 ± 1048
LTC_4	15726 ± 2671	262626 ± 27123	5194 ± 956
LXA_4	15042 ± 1300	266017 ± 27033	6212 ± 820
LXB_4	12610 ± 3135	259149 ± 10066	5688 ± 1367
12-HETE	13333 ± 2491	292534 ± 29602	5069 ± 925
15-HETE	14730 ± 2491	279571 ± 29602	6315 ± 940

Cells (5×10^5) were grown for 3 days in 10% FCS in the presence or absence of 1 μM of AA, LTB_4, LTC_4, LXA_4, LXB_4, 12-HETE and 15-HETE. Results (in dpm) are the mean ± S.E.M. from four independent experiments in n=6 samples.

The addition of lipoxygenase metabolite of AA is reported to modulate the growth of various cell types.[5–7] We then assessed their putative role on the proliferation of human myeloma cells. As reported in Table 2, the addition of micromolar amounts of AA, LTB_4, LTC_4, LXA_4, LXB_4, 12-HETE and 15-HETE has no significant (P>0.05, Mann–Whitney U-test, four independent experiments) effect on the [^{3}H]thymidine incorporation of U266, IM9 and OPM2 cells. This result is in accord with the fact that the enhanced B-cell proliferation caused by lipoxygenase blockade could not be reversed by the exogenous addition of LTs or HETEs.[2] In this study we have only investigated the effects of lipoxygenase products of AA on the growth of myeloma cells. However, cyclooxygenase products of AA such as prostaglandin E_2 are reported to modulate the proliferation of several cell types.[15,16] Clearly their role on the growth of U266, IM9 and OPM2 cells deserves to be clarified.

In conclusion, while human myeloma cells incorporate AA in their membrane phospholipids and triglycerides, exogenous AA and lipoxygenase products are not important modulators of their proliferation, a result that markedly differs to their reported endogenous role.

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