Altered expression and localization of 5-lipoxygenase accompany macrophage differentiation in the lung

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Covin, Randal B., Thomas G. Brock, Marc B. Bailie, and Marc Peters-Golden. Altered expression and localization of 5-lipoxygenase accompany macrophage differentiation in the lung. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L303–L310, 1998.—The alveolar macrophage (AM) exhibits a greater capacity to synthesize bioactive leukotrienes from arachidonic acid than does its circulating precursor the peripheral blood monocyte. Macrophage differentiation in the lung entails cellular residence within both the pulmonary interstitial and alveolar compartments. In this study, we sought to determine 1) whether this enhanced metabolic activity was acquired during maturation within the alveolar space and 2) the underlying mechanisms responsible for this upregulation. Rat AMs were separated by Percoll gradient centrifugation into four density-defined subpopulations thought to reflect their degree of maturation. On stimulation with a calcium ionophore, synthesis of leukotriene B₄ increased with the degree of maturation, although it was diminished in the oldest subpopulation. This maturation-dependent upregulation was not explained by increases in arachidonic acid release but was associated with increased expression of 5-lipoxygenase (5-LO) protein as determined by immunoblot analysis. Whereas 5-LO is primarily cytosolic in monocytes, it is known to be primarily intranuclear in unfractonated AMs. Here, the localization of 5-LO was investigated by immunofluorescence microscopy and was found to be predominantly nuclear in all AM subpopulations; by contrast, the protein was cytosolic in interstitial macrophages isolated by mechanical and enzymatic lung digestion. These divergent localization patterns in AMs and interstitial macrophages were verified in situ by immunohistochemical staining of sections of normal rat lung. When unfraccionated AMs were isolated and maintained in culture for 3 days, a shift in 5-LO distribution from nucleus to cytosol was observed. We conclude that 1) nuclear import of 5-LO occurs within the alveolar space and is reversible on removal from the alveolar milieu and 2) leukotriene synthesis capacity increases further during AM residence within the alveolar space as a result of a progressive increase in the amount of 5-LO protein.

\[ \text{specific differentiation (4). Because PBMs must migrate through the pulmonary interstitium en route to the alveolus, the interstitial } \text{Mφ (IM) is thought to represent an intermediate stage of differentiation between PBMs and AMs. Differentiation of AMs, therefore, encompasses events that occur in both the interstitial and alveolar compartments of the lung.} \]

Compared with most other cell types, Mφs are relatively enriched in arachidonic acid (AA) (23). This fatty acid can be hydrolyzed from membrane phospholipids and metabolized to a family of products known as eicosanoids, which exert diverse and potent effects on the processes of inflammation, tissue injury, and repair (25). The enzyme 5-lipoxygenase (5-LO) catalyzes the first two steps in the metabolism of AA to one group of eicosanoids, the leukotrienes (LTs). LTs play a pivotal role in both normal host defense and pathological states of inflammation (21). They have been shown to promote leukocyte chemotaxis and activation, vascular tone and permeability, smooth muscle contractility, immune function, and cell growth and differentiation. On the other hand, certain PGs, formed from the metabolism of AA via the cyclooxygenase pathway, tend to suppress inflammation and immune response (5, 19).

Interestingly, AMs have been shown to differ substantially from other nonpulmonary tissue Mφs (e.g., those isolated from the peritoneum, liver, brain, and pleura) as well as from pulmonary IMs and PBMs with respect to a number of cell functions. These include antigen presentation, phagocytic capacity, generation of oxidants, and elaboration of cytokines (16). Likewise, it has previously been shown that AMs metabolize AA preferentially via the 5-LO pathway to LTs, synthesizing only small amounts of PGs; by contrast, PBMs and nonpulmonary tissue Mφs metabolize AA to greater quantities of PGs than LTs (2, 3, 30, 36). It is essential that the AMs have the capacity to mount a rapid inflammatory response to maintain the sterility of the alveolar surface. This increased capacity for LT synthesis may therefore be essential for the AMs to meet the unique host defense requirements of the hostile pulmonary alveolar milieu (1). Not surprisingly, IMs appear intermediate between PBMs and AMs in their eicosanoid synthetic profile (30).

Studies to identify the possible molecular mechanisms underlying the increased LT synthetic capacity of AMs have revealed at least two differences between AMs and PBMs. First, AMs contain significantly greater levels of the two proteins that interact to carry out the 5-lipoxygenation of arachidonate, namely, the enzyme 5-LO and its helper protein 5-lipoxygenase-activating protein (FLAP) (11, 31). Second, 5-LO is located predomi-
nantly within the nucleus of resting AMs, whereas it is located primarily in the cytoplasm of PBMs (7, 38).

AMs are long-lived cells that are well known to exhibit substantial morphological and functional heterogeneity. Lavageable AMs can be separated by density into various subpopulations (17) thought to represent cells at different maturational stages, with the most dense (high nuclear-to-cytoplasmic ratio) AMs representing the youngest cells and the least dense (low nuclear-to-cytoplasmic ratio) cells representing the oldest. Differences in PG synthesis among density-defined AM subpopulations have been reported (9, 13). However, there is no information regarding differences in LT synthetic capacity, the amount of total 5-LO and FLAP, and the subcellular distribution of 5-LO in these AM subpopulations. The present study was undertaken to examine these characteristics of the 5-LO metabolic machinery throughout the stages of AM differentiation in the lung. This was accomplished by studying density-defined subpopulations of rat AMs as well as IMs.

MATERIALS AND METHODS

AM isolation. Specific pathogen-free female Wistar rats weighing 125–150 g were obtained from Charles River (Portage, MI) and housed under pathogen-free conditions. Resident AMs were obtained by lavage of the lungs as previously described (30).

Separation of AM subpopulations by Percoll gradient centrifugation. Isosmotic Percoll was formed by combining Percoll (Pharmacia Biotech, Uppsala, Sweden) with 10× PBS at 9:1 (vol/vol). Isosmotic Percoll was then diluted with PBS to a final concentration of 52%. Ten milliliters of 52% isosmotic Percoll were placed into a 16 × 76-mm (13.5-ml) thick-walled polycarbonate centrifuge tube (Beckman Instruments, Palo Alto, CA) and centrifuged at 20,300 g for 30 min at 4°C, and AM subpopulations were recovered in four density-defined fractions: fraction 1, <1.055 g/ml; fraction 2, 1.056–1.060 g/ml; fraction 3, 1.061–1.065 g/ml; and fraction 4, >1.065 g/ml.

Isolation of IMs. IMs were isolated with a method modified from Lavnikova et al. (20). Briefly, the lungs were perfused with 50 ml of ice-cold solution 1 (1.4 M NaCl, 50 mM KCl, 0.1 M HEPES, 60 mM glucose, and 25 mM NaPO 4 ). The lungs and trachea were excised and lavaged with 100 ml of 1.056–1.061 g/ml solution 1 from Lavnikova et al. (20). Briefly, the lungs were perfused with 30–60 min on ice with shaking. After this incubation, the mixture was filtered two times through the 15-µm mesh to remove granulocytes, and the remaining tissue was incubated with PBS-10% FCS-0.01% DNase containing 175 U/ml of collagenase D (Boehringer Mannheim). This mixture was incubated for 30–60 min on ice with shaking. After this incubation, the mixture was filtered two times through 15-µm mesh, with the filtrate containing the IMs.

Measurement of LT synthetic capacity in AMs. Unfractionated AMs (2 × 10 5) or density-defined subpopulations of AMs were suspended in 1 ml of medium 199 (M199; Graco BRL, Grand Island, NY) in wells of a 24-well plate (Falcon, Oxnard, CA). They were cultured at 37°C in a humidified atmosphere of 5% CO 2 in air. After 1 h, nonadherent cells were removed by washing two times with PBS, and cell monolayers were cultured overnight in M199 with 10% newborn calf serum (NCS). After overnight culture, monolayers have been found to contain ≥96% Mφs by morphology and nonspecific esterase staining (30).

The following morning, the cells were washed three times with PBS and incubated for 30 min at 37°C in M199 containing either 1 µM calcium ionophore A-23187 (Calbiochem, La Jolla, CA) or the diluent DMSO (final concentration 0.05%). After 30 min, the cell-free supernatant was removed and immunoreactive LTB 4 was quantitated by an acetylcholine-esterase-linked immunooassay kit from Cayman Chemical (Ann Arbor, MI) according to the manufacturer's instructions. Cell monolayers were harvested for total protein determination with a microtiter plate modification of the Coomassie dye-binding assay (Pierce, Rockford, IL) with BSA as a standard. Each condition was studied in triplicate, and LTB 4 synthesis is expressed as picograms of LTB 4 per microgram of total cellular protein.

Measurement of AA release from prelabeled AMs. AM subpopulations were plated at a density of 2 × 10 5 cells/ml in each well of a 24-well plate. The cells were allowed to adhere for 1 h as described in Material and Methods. Measurement of LT synthetic capacity in AMs, and cell lipids were prelabeled by including [ 3 H]-labeled AA during overnight culture in M199 with 10% NCS. After the overnight labeling period, the cells were washed three times with PBS containing 0.1% BSA to remove unincorporated label and stimulated with either 1 µM A-23187 or DMSO in M199–0.1% BSA for 30 min at 37°C. Albumin was included to trap all released [ 3 H]AA in the medium. Cell-free supernatants were analyzed for [ 3 H] by scintillation counting with a Beckman LS 1801 scintillation counter. Cellular uptake was determined by scintillation counting of cell lysates as previously described (28). All conditions were studied in triplicate, and AA release is expressed as a percentage of incorporated [ 3 H].

Immunoblot analysis of AM subpopulations. Homogenates of AM subpopulations were prepared as follows. The cells were resuspended in 200 µl of ice-cold sonication buffer (50 mM potassium phosphate, 100 mM NaCl, 1 mM dithiothreitol, 2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 60 µg/ml of soybean trypsin inhibitor, pH 7.1). They were sonicated with a Branson (Danbury, CT) sonifier at a constant duty cycle, setting 2, for 30 s. Aliquots from the four subpopulations containing equal amounts of protein (∼15–20 µg) were subjected to SDS-PAGE under reducing conditions with 12.5% polyacrylamide gels to separate 5-LO and FLAP. Proteins of interest were detected by immunoblot analysis as...
Density-defined subpopulations of AMs. Four rats were routinely subjected to lavage for the purpose of obtaining density-defined AM subpopulations. The total number of cells recovered after fractionation was $25.6 \pm 1.8 \times 10^6$ (n = 5 experiments), which represented $\sim 55\%$ of those AMs placed on the Percoll gradient. Fraction 1 represents the least dense (corresponding to the oldest) subpopulation, and fraction 4 represents the most dense (youngest) subpopulation. The distribution of cells in the four subpopulations was as follows: fraction 1, 31.0 ± 4.4%; fraction 2, 32.1 ± 4.5%; fraction 3, 16.9 ± 1.7%; and fraction 4, 20.0 ± 2.9% of recovered cells.

LTB$_4$ synthetic capacity of AM subpopulations. Synthesis of LTB$_4$, the major 5-LO metabolite of AMs (30), was assessed. AM subpopulations incubated with the diluent DMSO alone produced no discernible LTB$_4$ (data not shown). Maximal synthetic capacity was evaluated after stimulation with the potent agonist A-23187. LTB$_4$ synthetic capacity increased with decreasing cell density from fraction 4 to fraction 2 (Fig. 1). The least dense fraction, fraction 1, however, showed a substantial diminution of LTB$_4$ synthesis. The capacities of LTB$_4$ production were compared by Fisher's protected least significant difference test. The results are expressed as means ± SE from 3 individual experiments. *P < 0.05 vs. all other subpopulations.
ity of fraction 2 was statistically greater than that of each of the other fractions.

AA release in AM subpopulations. Because differences in LTB₄ synthetic capacity may represent differences in the activity of phospholipase A₂ (PLA₂), we estimated PLA₂ activity by measuring AA release from subpopulations of AMs. AMs incubated with DMSO alone showed no appreciable release of AA (data not shown). There were no significant differences in ionophore-stimulated AA release among the four subpopulations (fraction 2, 125 ± 13%; fraction 3, 117 ± 20%; and fraction 4, 155 ± 28%; n = 3 experiments), suggesting that the differences in LTB₄ synthetic capacity among them were not due to differences in PLA₂ activity. These results implicate a maturation-related change within the 5-LO pathway.

Total cellular 5-LO protein and FLAP in AM subpopulations. To discern whether the changes in LTB₄ synthetic capacity might be secondary to changes in total cellular 5-LO or FLAP, whole cell homogenates from the four AM subpopulations were subjected to immunoblot analysis for 5-LO. Figure 2 shows that the total amount of 5-LO protein increased from fraction 4 to fractions 3 and 2 and then decreased precipitously with fraction 1. 5-LO expression in both fractions 2 and 3 was statistically greater than that in either fraction 1 or 4. There did not appear to be any difference in total FLAP among the four subpopulations (Fig. 3). These findings demonstrate that changes in LTB₄ synthetic capacity during Mϕ residence in the alveolar space are associated with changes in the amount of total 5-LO but not of FLAP.

Subcellular distribution of 5-LO in AMs and IMs. Because it is known that the predominant subcellular locale of 5-LO shifts from the cytosol to the nucleus as the PBM differentiates into a mature AM, we investigated whether this change occurs within the alveolar space. In all four density-defined subpopulations, immunofluorescence microscopy of AMs examined ex vivo demonstrated 5-LO to be predominantly intranuclear (Fig. 4). These results suggest that the change in the subcellular distribution of 5-LO occurs either within the pulmonary interstitium or very soon after entry into the alveolar space. To assess whether this change in the subcellular distribution of 5-LO occurs within the pulmonary interstitium, cells were isolated from rat lung digests as described. ED1 and ED2 staining was used to identify cells in the interstitial cell preparation as AMs and IMs, respectively (12, 35). Figure 5A shows AMs in the preparation staining positively for ED1, with its expected cytosolic pattern and with a predominantly nuclear pattern of 5-LO staining. By
contrast, Fig. 5B shows IMs staining positively for ED2, with its expected plasma membrane pattern and with a cytosolic pattern of staining for 5-LO. To verify the subcellular distribution patterns observed in AMs and IMs examined ex vivo, we performed immunohistochemical analysis of 5-LO in sections of normal rat lung to evaluate staining patterns of the two cell populations in situ. A predominant intranuclear distribution of 5-LO was consistently observed in intra-alveolar Mφs from multiple lung sections (Fig. 6A). Although most cells of the alveolar walls (presumably epithelial cells) were 5-LO negative, occasional positively staining cells were identified in alveolar walls, and these had a cytosolic distribution (Fig. 6B); these are presumed to represent IMs. Taken together, these findings suggest that the change in subcellular distribution of 5-LO does not occur in the pulmonary interstitium but occurs very early on entry into the alveolar space.
Shift of 5-LO compartmentalization during culture of AMs. Because nuclear import of 5-LO appeared to be a consequence of MΦ ingress into the alveolus, we next wished to determine whether, on removal of AMs from the alveolar milieu, 5-LO would move out into the cytosol. In contrast to the predominantly intranuclear distribution of 5-LO in AMs examined in situ (Fig. 6A) or in unfractionated AMs examined by immunofluorescence microscopy shortly after retrieval (Fig. 7A), AMs maintained in culture for 3 days exhibited a predominant cytosolic distribution of 5-LO, with very little intranuclear protein (Fig. 7B). Thus the accumulation of 5-LO within the nucleus, which occurs on MΦ entry into the alveolar space, is reversible on their removal from that environment.

**DISCUSSION**

Changes in both 5-LO expression (11, 31) and compartmentalization (7, 38) are known to accompany MΦ differentiation in the lung, and these represent mechanisms that might contribute to the increased LT synthetic capacity of AMs compared with that of PBMs (2, 3). The present study sought to determine whether these alterations occurred during the interstitial or alveolar phases of pulmonary MΦ differentiation. We found that the change in subcellular distribution of 5-LO from a predominantly cytoplasmic locale in PBMs to a predominantly nuclear locale in resting AMs takes place on entry into the alveolar space rather than in the pulmonary interstitium. In addition, LT synthetic capacity continues to increase while the AM matures within the alveolar space as a result of a continued increase in the amount of total 5-LO protein.

5-LO has recently been shown to be localized to the cytosol of resting PBMs (38) and the nucleus of resting AMs (7, 38). At what stage in the continuum of AM differentiation this change took place, however, was not known. Here we present evidence that this change in subcellular distribution takes place within the alveolar space. First, we used a procedure that, by extensively removing contaminating AMs (20), has been demonstrated to obtain a population of IMs substantially purer than that previously available (22). Using the monocyte-macrophage antibodies ED2 and ED1 (12) to distinguish IMs from contaminating AMs, respectively (35), we performed dual staining with an antibody to 5-LO. Our results indicate that 5-LO is absent from the nucleus of IMs, whereas it is present within the nucleus of AMs. Second, density-defined subpopulations of lavaged AMs show nuclear localization of 5-LO even within the youngest cell population. Third, 5-LO was found to be largely intranuclear in virtually all the intra-alveolar MΦs visualized in situ by immunohistochemical staining of normal rat lung tissue. This confirms recent results obtained with human lung tissue (37) and also reduces the likelihood that the ex vivo localization data are artifacts of the isolation procedures. A precedent for rapid shifts in 5-LO compartmentalization, such as that which might occur on entry of pulmonary MΦs into the alveolar space, can be found in a recent report by Brock et al. (6) that 5-LO can redistribute rapidly from the cytosol to the nucleus during in vivo recruitment or in vitro adherence of peripheral blood neutrophils. The possibility that nuclear import of 5-LO during differentiation of AMs is the consequence of exposure to the alveolar milieu is further supported by our observation that the protein resumes a predominantly cytosolic distribution on AM removal from the lung and culture ex vivo for an extended time period.

The LT synthetic capacity of AMs is significantly higher than that seen in PBMs or other tissue MΦs (e.g., peritoneal MΦs and pleural MΦs) (2, 3, 29). Where in the lung this metabolic change takes place has not been determined. A previous report by Peters-Golden et al. (29) showed the LT synthetic capacity of IMs to be intermediate between those of PBMs and AMs; however, this “IM” preparation was assuredly contaminated with AMs, suggesting that the LT synthetic capacity of IMs was more likely much closer to PBMs than to AMs. Although the recovery of IMs with the isolation procedure described herein did not yield sufficient cells to allow metabolic or immunoblot studies, we were able to evaluate density-defined subpopulations of AMs to assess whether there was an increase in AA release, LT synthetic capacity, or total 5-LO and FLAP as AMs mature within the alveolar space.

It has been well documented that AMs are not a homogeneous population of cells (18). Density-defined subpopulations of AMs have been reported to differ with respect to ultrastructural features (24) as well as to a number of functional properties including plasminogen activator activity (33), interleukin-1 production (14, 26, 32), and superoxide anion production (27). In addition, differences in PG production have also been identified in subpopulations of AMs (9, 13). Our present results are the first to demonstrate differences in LT synthetic capacity and total 5-LO protein in AM subpopulations. Specifically, as AMs mature within the alveolar space, their LT synthetic capacity increases as a result of an increase in total 5-LO protein. This increase occurs from fraction 4 (the most dense or youngest fraction) through fraction 2. Fraction 1 (the least dense or oldest fraction) shows a large decline in

![Fig. 7. Effect of removal of AMs from alveolus on intracellular distribution of 5-LO as determined by immunofluorescence microscopy. A: unfractionated AMs obtained by lavage and adhered to plastic for 5 min. Note a predominant intranuclear pattern of fluorescence. B: unfractionated AMs adhered and cultured ex vivo for 3 days. Note a predominant cytosolic pattern of fluorescence.](image-url)
both LT synthetic capacity and total 5-LO protein. It is possible that this fraction represents a senescent population of AMs with respect to LT production. A similar decrease among the lowest density fraction has also been reported for production of the prostanoids PGE2 and thromboxane A2 (9), interleukin-1 release (26, 32), and Ia antigen expression and chemotactic migration (26). In our study, arachidonate release and total FLAP did not significantly differ among density-defined subpopulations of AMs. This suggests that the numerous changes that take place as PBMs mature into AMs with respect to arachidonate metabolism via the 5-LO pathway do not take place at the same time and at the same location. Further evaluation of IM, cut, and disaggregated cell populations should help to clarify the spectrum and relative importance of changes that occur as PBMs move through the pulmonary interstitium and enter the alveolar space. The specific microenvironmental signals that elicit these changes also remain to be elucidated.

In addition to the increased 5-LO protein expression that occurs during Mδ differentiation in the alveolar space, the nuclear import of 5-LO might itself contribute to the increased LT synthetic capacity of AMs. Previous studies by Balter et al. (2) and Peters-Golden et al. (30) with various mononuclear phagocyte populations from rats and humans are certainly consistent with that possibility because AMs from both species (with nuclear 5-LO) have a greater threshold for agonist stimulation and a greater maximal LT synthetic capacity than do PBMs, IMs, or peritoneal Mδs (all of which have cytosolic 5-LO). More convincing evidence that this is a causal relationship derives from the recent finding by Brock et al. (6) that nuclear import of 5-LO, which occurs rapidly during the process of neutrophil recruitment from the bloodstream to the peritoneum, was associated with increases in the threshold concentration for agonist stimulation as well as in the maximal capacity for LT generation. Interestingly, it has long been known that ex vivo culture over 3 days is accompanied by an appreciable decline in the LT synthetic capacity of AMs (34). That culture of these cells over an identical time frame is now demonstrated to result in a shift in 5-LO compartmentalization from nucleus to cytosol further supports the possibility that the nuclear distribution of 5-LO per se contributes to the high LT synthetic capacity of AMs. The mechanisms that might underly such a phenomenon are not clear at the present time.

In summary, the present study demonstrates that the nuclear import of 5-LO that occurs during the process of AM differentiation in the lung takes place within the alveolar space. This phenomenon, which is reversed when AMs are removed from the alveolar space and cultured ex vivo, may itself increase the metabolic function of the enzyme. In addition, LT synthetic capacity continues to increase while the AM matures within the alveolar space as a result of a progressive increase in the amount of total 5-LO protein. These changes, which result in an enhanced capacity to metabolize AA via the 5-LO pathway, would be expected to amplify the antimicrobial host defense capabilities of the resident AMs (1).

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