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Fetal hepatic expression of 5-lipoxygenase activating protein is confined to colonizing hematopoietic cells

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ABSTRACT

Leukotriene C_4 is a potent inflammatory mediator formed from arachidonic acid and glutathione. 5-Lipoxygenase (5-LO), 5-lipoxygenase activating protein (FLAP) and leukotriene C_4 synthase (LTC_4S) participate in its biosynthesis. We report evidence from *in situ* hybridization experiments that FLAP mRNA is abundantly expressed in fetal mouse liver from e11.5 until delivery. In contrast very little or no FLAP mRNA was detected in adult liver. The fetal expression in liver was not uniform but occurred in patches. Cells from e15.5 livers were fractionated by fluorescence activated cell sorting into hepatocytes and other $CD45^-$ cells and $CD45^+$ hematopoietic cells. The latter were further separated into immature (Lin^-) and mature (Lin^+) cells and analyzed for FLAP mRNA content by quantitative RT-PCR. FLAP mRNA expression was confined to $CD45^+$ cells and the mature cells had approximately 4-fold higher FLAP mRNA levels compared to the immature cells.

The membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamily includes (among 136 members): microsomal glutathione transferases (MGST) 1, 2 and 3, microsomal prostaglandin E synthase 1 (mPGES1), 5-lipoxygenase activating protein (FLAP), and leukotriene C_4 synthase (LTC_4S) [1]. In addition to 5-lipoxygenase (5-LO), the two latter MAPEG proteins are required for the formation of the potent, pro-inflammatory cysteinyl leukotrienes (LT) [2,3]. FLAP facilitates the transfer to 5-LO of arachidonic acid, released from membrane phospholipids by phospholipase A_2 [4], and renders 5-LO capable of converting endogenous arachidonic acid to LTA_4 [5] whereas LTC_4S catalyzes the conjugation of LTA_4 to glutathione [6,7]. Recently, a resistance locus to atherosclerosis in LDL receptor knock-out mice was mapped to the 5-LO gene; the resistant mice had markedly decreased expression of 5-LO mRNA [8]. In man 5-LO mRNA and protein expression in atherosclerotic lesions correlated with the severity of the disease [9]. Another study in man demonstrated a four marker SNP in the FLAP gene with double risk of developing myocardial infarction and nearly doubled risk of having stroke [10]. Moreover, G/A polymorphism at position −1072 of the LTC_4S promoter raised the incidence of ischemic cerebrovascular disease whereas A/C polymorphism at position −444 lowered it [11]. In addition to expression in cells of myeloid origin [12] FLAP expression has also been observed in adipocytes and other cellular components of surgically removed (for cosmetic reasons) adipose tissue and proposed to have relevance for obesity and insulin resistance [13].

Materials and methods

Materials. Antibodies for cell surface staining were from Biolegend, San Diego, CA [PECY5-CD19 (RA3–6B2), GR-1 (RB6–8C5)]; BD-Biosciences, San Jose, CA [CD11b/MAC1 (M1/70), CD3 (17A2), CD32/16]; or eBioscience, San Diego, CA [TER-119 (LY-76), CD45-PECY7(30-F11)]. [α-^{35}S] UTPαS was from PerkinElmer, Boston, MA; MAXI script *in vitro* transcription kit for probe preparation was from Ambion, Austin, TX and quick spin columns for probe purification were from Roche Diagnostics, Indianapolis, IN. SuperScript III first strand synthesis super mix for quantitative RT-PCR and propidium iodide were from Invitrogen, Paisley, UK; mastermix for qRT-PCR, and FLAP (Mm00802100_m1) plus HPRT1 (Mm00446968_m1) assay mixes were from Applied Biosystems, Foster City, CA; RNeasy micro kit was from Qiagen, Düsseldorf, Germany, and RPN40 (LM1) photographic emulsion was from GE-Healthcare, Amersham, UK. All other materials were from sources described before [14,15]. cDNA encoding murine FLAP was purchased from Geneservice, Cambridge, UK (clone id 1349819).

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Animal experiments. All experimental procedures were approved by the Animal Care and Use Committee at Linköping University. NMRI mice from Scanbur BK, Sollentuna, Sweden were housed one to a cage in a pathogen-free facility at 20 °C with a regular 12 h light/dark cycle (lights on 7:00 A.M. to 7:00 P.M.). Food and water were provided *ad libitum*. Animals were euthanized with 70% v/v CO_2 in air prior to removal of fetuses or organs.

In situ hybridization. cDNA encoding murine FLAP was amplified by PCR using 5′-AGCCTGGATCCCTCTGGCGGG-3′ and 5′-CGTCTCTCTAGATCTGGTGAGCGTCC-3′ as forward and reverse primers, respectively and subcloned into BamHI and XbaI sites of a pcDNA3 expression vector. Probes were synthesized using SP6 RNA polymerase for antisense and T7 RNA polymerase for sense probe in the presence of [α-^{35}S] UTPαS according to the MAXI script protocol and purified on quick spin columns. NMRI mouse embryos at various stages (noon on the day of appearance of the vaginal plug being e0.5) were fixed in 4% (w/v) formaldehyde. Adult tissues were dissected from NMRI mice perfused with 0.9% (w/v) NaCl and fixed in 4% (w/v) formaldehyde. Fixed embryos and adult tissues were treated with 20% w/v glucose in PBS and cryo-sectioned sagittally at 14–18 μm thickness. Whole-mount *in situ* hybridization was performed as described [16]. Tissue sections were hybridized to [^{35}S]-labeled probes and washed. RNA hybrids were detected by autoradiography using X-ray film or photographic emulsion. After being developed emulsions were examined by dark-field microscopy.

Sorting of fetal liver cells. Nine livers from e15.5 embryos were pooled in PBS with 5% (v/v) fetal bovine serum, a single cell suspension was obtained by using a 5-ml syringe with a 25G needle. The cells were then filtered through a 70 μm cell strainer to remove cell aggregates. The single cell suspension obtained was stained with CD45-PECY7 (1 μg/ml) and a linage cocktail of PECY5-CD19 (1 μg/ml), GR-1 (1 μg/ml), CD11b/MAC1 (0.5 μg/ml), CD3 (0.5 μg/ml), and TER-119 (2 μg/ml) after incubating the cells with CD32/16 (Fc blocker, 4 μg/ml) antibodies. Propidium iodide (0.5 μg/ml) was added to discriminate dead from viable cells. The stained cells were sorted into CD45$^-$ cells, CD45$^+$/Lin$^+$ cells and CD45$^+$/Lin$^-$ cells using a Becton–Dickinson FACS (fluorescence-activated cell sorter), Aria SORP. After purity checking of all indicated cell populations, the cells were sorted directly to Eppendorf tubes, each containing 350 μl RLT lysis buffer (Qiagen RNA isolation kit) and frozen on dry ice immediately after sorting.

RT-PCR. Total RNA was isolated from e15.5 livers using an RNeasy micro kit. cDNA was prepared using a Superscript III kit and analyzed by PCR with FLAP specific primers (forward: 5′-GGTCTACACTGCCAACCAGAACTG-3′; reverse: 5′-CCGAAGAAGAA-GATGAGGTAATGG-3′).

Quantitative (q)RT-PCR. RNA was isolated from unsorted e15.5 fetal liver cells and sorted cell populations and each sample (50 ng) was converted to cDNA using Superscript III. FLAP expression was analyzed by qRT-PCR with HPTR1 as control gene using Applied Biosystems 7500 Real-Time PCR system and the TaqMan probe protocol.

Results

Liver is a major site of FLAP mRNA expression during mouse embryogenesis

In order to investigate the expression pattern of FLAP message during mouse embryogenesis whole body sections of embryos at different stages of development were examined by *in situ* hybridization using [^{35}S]-labeled FLAP antisense probe. Autoradiography showed abundant FLAP mRNA expression in the fetal liver already at e11.5 (Fig. 1A). From this stage until e17.5 liver was the only site where FLAP expression was observed. At e17.5 and e18.5 expression was also detected in some other organs, notably spleen, thymus and vertebrae (Fig. 1A). Expression at these other sites was also observed in neonatal mice (data not shown).

Analyses by dark-field microscopy of liver sections dipped in photographic emulsion revealed non-homogeneous expression of FLAP message in patches throughout the liver. These patches became more and more separated with increasing liver size (Fig. 1B). Although weaker, it was still detected for some time after birth. Adult liver, however, had no detectable FLAP expression. To verify the finding of FLAP mRNA expression in fetal liver, total RNA isolated from e15.5 unsorted liver cells was analyzed by RT-PCR using FLAP specific primers (Fig. 1C). A negative control without template and a positive control with FLAP cDNA as template were

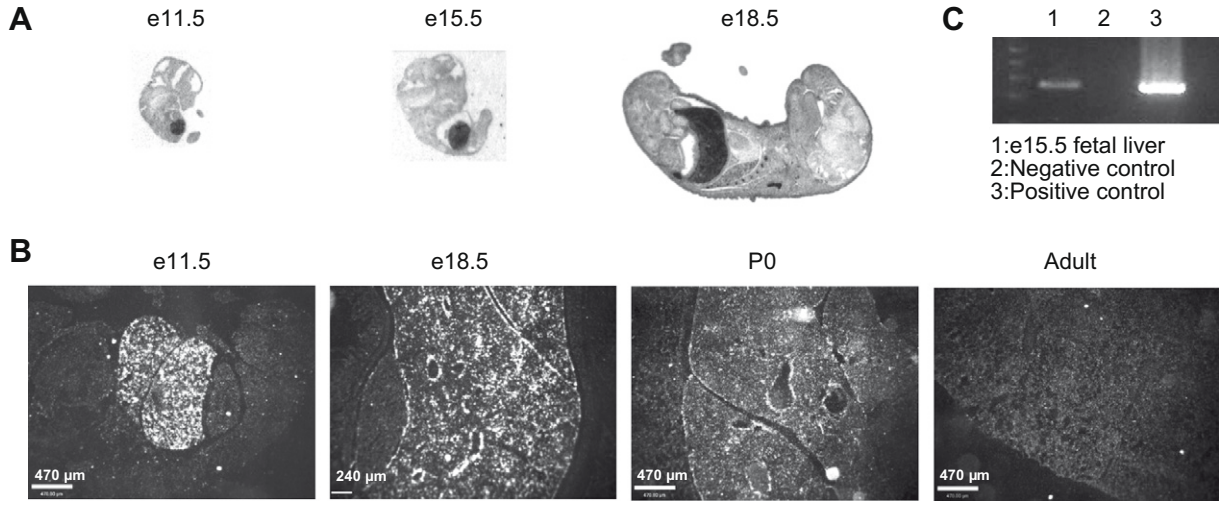

Fig. 1. Expression of FLAP mRNA in fetal liver. Sagittal sections of mouse embryos were hybridized with FLAP antisense and sense (not shown) mRNA probes. Expression was observed in the liver from e11.5 and until birth. No or minimal expression was observed in adult liver. (A) *In situ* hybridization detected by autoradiography in sagittal sections of whole embryos. (B) *In situ* hybridization of fetal liver at different stages of development analyzed by dark-field microscopy after development of coating photographic emulsion. (C) Detection of FLAP mRNA expression in e15.5 mice fetal liver by RT-PCR. Negative control, no template; positive control, FLAP cDNA as template.