The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/25768

Please be advised that this information was generated on 2019-08-05 and may be subject to change.
Endothelial Cells of the Human Microvasculature Express Epidermal Fatty Acid-Binding Protein

Isabelle Masouye, Gerry Hagens, Toin H. Van Kuppevelt, Peder Madsen, Jean-Hilaire Saurat, Jacques H. Veerkamp, Michael S. Pepper, Georges Siegenthaler

Abstract  Epidermal fatty acid-binding protein (E-FABP), previously characterized in human keratinocytes, is a cytoplasmic protein of 15 kD that specifically binds fatty acids (FAs). Previous PAGE-immunoblotting studies indicated that several human tissues display an immunoreactive band with an electrophoretic mobility identical to that of E-FABP. The aim of this study was to determine in which cells, other than keratinocytes, E-FABP might be expressed. By immunohistochemistry, we show that E-FABP is expressed in endothelial cells of the microvasculature of the placenta, heart, skeletal muscle, small intestine, lung, and renal medulla. Interestingly, in lung, a tissue of endodermal origin, E-FABP staining was also localized to secretory cells, ie, Clara cells, goblet cells, and probably a subpopulation of pneumocytes. RNA isolated from cultured human umbilical vein and normal human dermal microvascular endothelial cells was analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR). Southern blotting and sequencing of the cloned RT-PCR products demonstrate that endothelial E-FABP is identical to keratinocyte E-FABP. These data suggest that E-FABP-mediated FA transport occurs at the level of the microvasculature in several FA target organs. (Circ Res. 1997;81:297-303.)

Key Words  • fatty acid  • blood vessel  • heart  • placenta  • lung

The FABPs constitute a superfamily of highly conserved 14- to 15-kD cytosolic lipid-binding proteins. These specific carriers are widely distributed among different cell types and are involved in the transport of long chain FAs. Several observations suggest that the major function of FABPs is to regulate intracellular levels of FAs and their targeting to specific metabolic pathways.1-3 FAs are important molecules for membrane formation, energy delivery, and lipid mediation. It has recently been suggested that FABPs may also interact with several FA-mediated cellular processes, such as control of cell growth, cell signaling, and regulation of gene transcription (reviewed in References 2, 4, 5, and 6). At least eight FABP types can be discriminated and have been named after the first tissue in which they were identified.1-3 Each FABP type displays a characteristic pattern of tissue expression and distinct ligand-binding properties, suggesting specific and complementary functions for each protein.

We previously characterized E-FABP in human epidermis and showed that it specifically binds FAs, with high affinity for stearic and linoleic acids.7,8 Immunohistochemical studies revealed that E-FABP is expressed during keratinocyte differentiation in human oral mucosa, in human epidermis both under normal and pathological conditions, and in cultured keratinocytes.5,9 PAGE-autoradioblotting and PAGE-immunoblotting studies of various human tissues revealed that E-FABP expression is not restricted to keratinocytes, since heart, intestine, and adipose tissue showed an immunoreactive band with an electrophoretic mobility identical to that of E-FABP from keratinocytes.7,8 To date, the cellular localization of E-FABP in nonepithelial tissues is unknown. Furthermore, it has not been established whether E-FABP from keratinocytes is identical to the immunoreactive band detected in other tissues.

In the present study, we show that E-FABP is expressed in nonciliated cells of the bronchial epithelium and in endothelial cells of the microvasculature of several tissues. By molecular cloning, we show that endothelial E-FABP is identical to keratinocyte E-FABP. The possible role of E-FABP in blood capillary transport function is discussed.

Materials and Methods

Tissue Specimens

Specimens from human heart, small intestine, skeletal muscle (quadriceps), lung, and kidney were obtained from autopsies (Department of Pathology, University Hospital, Geneva, Switzerland) and from biopsies obtained during surgery. Placenta and umbilical cord specimens were obtained immediately after obstetrical delivery.

Immunohistochemistry

Tissue samples were fixed for at least 24 hours in 10% formalin or Duboscq-Brazil solution and processed for routine histology. None of the tissues showed obvious pathological alterations by conventional hematoxylin-eosin staining. Rabbit antiserum against human E-FABP purified from psoriatic scales was obtained as described previously.8 The antiserum was specific, since for various tissues, SDS-PAGE and PAGE-
immunoblotting analysis showed only one immunoreactive band at 15 kD and Rf (relative mobility) 0.32, respectively. 

Paraffin sections (5 μm) were permeabilized in 0.3% Triton X-100 solution and preincubated with normal goat serum to block nonspecific binding sites. After incubation for 60 minutes at room temperature with the anti-E-FABP antiserum (dilution, 1:200), an immunoperoxidase technique using a biotinylated goat anti-rabbit IgG as secondary antibody and a peroxidase-conjugated avidin-biotin complex was applied according to the manufacturer's instructions (Vectorstain reagents, Vector Laboratory). Peroxidase reactivity was then revealed using hydrogen peroxide and diaminobenzidine as a chromogenic substrate. The sections were lightly counterstained with hematoxylin. As a control, preimmune serum from the immunized rabbit was used simultaneously on adjacent sections.

We performed comparative stainings on adjacent sections using vWF and UE1 as endothelial markers, together with a standard immunoperoxidase technique. vWF was detected using a rabbit antiserum directed against human vWF (factor VIII-related antigen, Dakopatts). Biotinylated swine anti-rabbit immunoglobulins (E353, Dako A/S) and a peroxidase-conjugated streptavidin-biotin complex (StreptABComplex/HRP, Dako A/S) were then applied according to the manufacturer's instructions. For UE1 staining, we used a biotinylated UE1 (Vector Laboratories) and a peroxidase-conjugated streptavidin-biotin complex (StreptABComplex/HRP, Dako A/S). The peroxidase was localized by the diaminobenzidine—hydrogen peroxide reaction, and the sections were lightly counterstained with hematoxylin.

Endothelial Cell Cultures

HUVECs

HUVECs isolated by collagenase digestion were provided by Dr N. Maggioni (University of Rome, Italy) and were cultured in gelatin (1.5%)-coated tissue culture dishes or flasks (Falcon Labware, Becton-Dickinson) in medium 199 (GIBCO BRL, Life Technologies) supplemented with 20% fetal calf serum (GIBCO BRL), 4 mmol/L L-glutamine, 30 μg/mL endothelial cell growth supplement (Collaborative Research), 100 μg/mL heparin (sodium salt, grade I, from porcine intestinal mucosa, Sigma Chemical Co), and antibiotics.

DMVECs

Normal DMVECs isolated from foreskin (Invitrocyte Inc) were grown in MEM (α modification, GIBCO BRL) and 10% donor calf serum (GIBCO BRL).

PAGE-Immunoblotting Analysis

Nonpathological heart and small intestine biopsies as well as cultured endothelial cell lines (HUVECs and DMVECs) were analyzed for E-FABP expression by PAGE-immunoblotting techniques under non-denaturing conditions as described previously. 10 Purified E-FABP a was used as a standard. Rabbit antiserum against human E-FABP was used to detect E-FABP. Similar experiments were performed using antisera directed against H-FABP and L-FABP. 11

Cloning of E-FABP From Human Endothelial Cells

RNA Isolation and cDNA Synthesis

Total cellular RNA from HUVECs and DMVECs was isolated according to the method of Chomczynsky and Sacchi. Poly(A+) mRNA was isolated from 2 μg total cellular RNA using a Dynabeads mRNA Direct kit (Dynal) according to the manufacturer's instructions. First-strand cDNA synthesis was directly carried out on Dynabead-captured Poly(A+ mRNA using 200 U M-MLV reverse transcriptase purchased from GIBCO BRL and 0.5 mmol/L dNTPs from Pharmacia in a total volume of 20 μL.

PCR

The cDNA mix was diluted 1:10, and 1 μL was used to perform a PCR. Since there is evidence that E-FABP is identical to the PA-FABP cloned by Madsen et al, 13 the following primers based on the PA-FABP sequence were purchased from Amplimun AG: sense primer (primer 1), 5'-ATGGGCCCACTTGCCAAGCTG-3' (position 48 to 69 of the PA-FABP cDNA); anti-sense primer 1 (primer 2), 5'-CAGGTTGACATTTGCATGAC-3' (position 408 to 428 of the PA-FABP cDNA); and anti-sense primer 2 (primer 3), 5'-TGCTTGAACGTAGGCTTG-3' (position 499 to 520 of the PA-FABP cDNA). PCR was carried out in 20 μL Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs each, and 0.25 μmol/L sense primer and anti-sense primer 1 or 2. After a denaturing step (3 minutes at 94°C), 2.5 U Taq polymerase from GIBCO BRL was added during a cooling step of 10 minutes at 80°C. Thirty-five PCR cycles were performed according to the following scheme: 1-minute denaturing at 95°C, 1-minute annealing at 50°C, and extension for 1.5 minutes at 72°C. After the last cycle, a final extension was performed at 72°C for 10 minutes.

Southern Blotting, Cloning, and Sequencing of PCR Products

The amplified cDNA fragments were separated in a 1.5% agarose gel and detected by ethidium bromide staining (0.5 μg/mL). A 100-bp DNA ladder from GIBCO served as a molecular standard. Transfer of separated DNA onto a Hybond N filter (Amersham) and prehybridization were performed as described previously. 14 A full-length PA-FABP cDNA was labeled using the Prime-a-Gene labeling kit from Promega according to the manufacturer's instructions. Hybridization with the labeled cDNA probe was performed overnight at 65°C. The filter was washed first at room temperature in 2× SSC/0.1% SDS for 30 minutes and 0.5× SSC/0.1% SDS for 30 minutes and then at 60°C in 0.1× SSC/0.1% SDS for 30 minutes and subsequently exposed to x-ray film. Cloning of the PCR products was done using the TA-cloning kit from Invitrogen according to the manufacturer’s instructions. Plasmids were isolated, and inserts were subsequently sequenced using the T7 sequencing kit from Pharmacia.

Results

Immunolocalization of E-FABP in Human Tissues

Controls

No staining was observed with preimmune serum in any of the tissue samples analyzed. A representative control experiment using placental tissue is shown in Fig 1A. Cross-reactivity with intestinal FABP, L-FABP, and H-FABP types could be excluded, since these display specific tissue localizations that differ from that of E-FABP. 4,15-18 Comparative stainings with either fixation...
method (10% formalin or Duboscq-Brazil solution) re­
vealed no significant differences.

**Placenta at Term**

Trophoblastic cells lining the placental villi were im­
munonegative. The endothelium stained strongly for E-FABP in a high proportion of fetal microvessels, including capillaries and venules (estimated to be >85% of vascular cross sections) (Fig 1B). Endothelium of small veins was occasionally positive.

**Skeletal Muscle**

Muscle cells from quadriceps did not express E-FABP. In contrast, the endothelial cells from the microvasculature surrounding the muscle fibers were clearly positive (Fig 1C).

**Heart**

No significant staining was observed in cardiomyo­
cytes or endocardial endothelial cells, but the microvas­
cular endothelium expressed E-FABP (Fig 1D).

**Small Intestine**

The epithelium lining the intestinal crypts and villi was negative for E-FABP, whereas capillaries localized in these villi were strongly immunoreactive (Fig 1E). Venules identified in the mucosa were negative. The microvasculature was positive in the submucosa (not shown).

**Adipose Tissue**

In adipose tissue of the intestinal wall, heart, and hypodermis, capillaries surrounding adipocytes as well as small collecting venules (Fig 1F) always exhibited intense immunostaining. On these paraffin-embedded sections, adipocytes did not demonstrate significant labeling (not shown).

**Umbilical Cord**

Umbilical cord vasculature consists of two arteries and a single vein that are devoid of vasa vasorum; E-FABP
Kidney vessels (Fig 1G and 1H) were immunonegative. 300 Circulation Research

lium lining the umbilical cord expressed E-FABP (not shown).

Smooth Muscle
Smooth muscle cells in the intestinal wall and in blood vessels (Fig 1G and 1H) were immunonegative.

Liver
No significant reactivity was detected in liver (not shown).

Kidney
The renal excretory system was immunonegative. In the cortex, the great majority of the vessels, including glomeruli and their afferent and efferent arterioles, were not stained (Fig 2A); only rare capillaries were faintly positive (estimated at <5% compared with UEA1 staining). In the medulla, E-FABP was detected in the endothelium of a subpopulation of peritubular capillaries (estimated at 80% compared with UEA1 staining). Vasa recta were negative (Fig 2B).

Lung
In bronchial epithelium, ciliated cells were immunonegative, whereas goblet cells expressed E-FABP (Fig 3A). E-FABP was also detected in a subpopulation of alveolar epithelial cells, most probably type 2 pneumocytes (Fig 3B). The peribronchial microvasculature was positive, and the alveolar capillary network was partially

Fig 2. Immunostaining for E-FABP and UEA1 on serial sections of the kidney. Endothelial cells in cortical vasculature were negative for E-FABP, including peritubular capillaries, glomeruli (arrow), and artery (star) (A); in the medulla, the endothelium was E-FABP positive in the majority of peritubular capillaries but was negative in vasa recta (arrows) (B). In contrast, all endothelial cells were positive for UEA1 both in cortex (C; star indicates artery; arrow, glomerule) and medulla (D; arrows indicate vasa recta). Scale bar=25 μm.

Fig 3. Localization of E-FABP in human lung. Goblet cells in bronchial epithelium (A) stained strongly for E-FABP. A subpopulation of pneumocytes (B) were also positive (arrows), and the capillary alveolar network was partially labeled (arrowheads) (B). Clara cells in terminal bronchioles (C) were also positive; panel D is a higher magnification of the boxed area in panel C. Note the absence of staining of the large vessel (arrow) in panel C. Bar=25 μm.
Comparative Study of E-FABP, vWF, and UEA1 in Endothelial Cells of Various Tissues

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Arteries</th>
<th>Capillaries</th>
<th>Venules</th>
<th>Veins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E-FABP</td>
<td>vWF</td>
<td>UEA1</td>
<td>E-FABP</td>
</tr>
<tr>
<td>Placenta†</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heart†</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quadriceps</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>Lung</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>Ileal mucosa</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Kidney†</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>Cortex</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Medulla</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>−</td>
</tr>
</tbody>
</table>

+/− indicates partially positive.
*The percentage of microvessels positive for E-FABP, vWF, and UEA1 was 85%, 75%, and 40%, respectively (estimated by vascular cross sections).
†More than 85% of the cortical microvasculature, including glomeruli, was negative for E-FABP. In renal medulla, 80% of the peritubular capillaries were positive for E-FABP (compared with UEA1 staining).

stained (Fig 3B). Clara cells in terminal bronchioles were also labeled (Fig 3C and 3D).

Comparative Stainings of E-FABP, vWF, and UEA1 in Various Organs

vWF and UEA1 were used to stain endothelial cells in order to compare their distribution with E-FABP. These data, summarized in the Table, revealed that E-FABP, vWF, and UEA1 had distinct expression patterns in endothelial cells from different tissues.

Depending on the organ studied as well as the localization of the vasculature in each organ, a variable number of capillaries and venules were strongly E-FABP positive. Although small arterioles were relatively difficult to identify on routine histological sections, E-FABP was not detected in this compartment of the microvasculature. Pericytes did not express E-FABP (Fig 1F).

As a general rule, in all organs studied, E-FABP was not detected in large blood vessels, including arteries, arterioles with internal elastic lamina, and large veins. Endothelial cells of small veins were only occasionally stained.

A representative experiment comparing E-FABP and UEA1 expression in kidney vasculature is illustrated in Fig 2.

Expression of E-FABP in Human Endothelial Cells

When protein extracts from intestine (Fig 4A, lanes 1 and 2), heart (lanes 3 and 4), HUVECs (lanes 5 and 6), and DMVECs (lanes 9 and 10) (20 μg and 40 μg loaded for each sample) were analyzed by PAGE-immunoblotting with antiserum directed against E-FABP, the immunoreactive band detected in these samples showed an electrophoretic mobility identical to purified E-FABP (lane 7 [0.5 μg] and lane 8 [0.01 μg]), suggesting that E-FABP from the various tissues and cell lines is similar to E-FABP from keratinocytes. By comparing the intensity of the purified E-FABP bands with bands from other samples, it was estimated that the amount of E-FABP per total protein ranges between 0.5% and 2%. Experiments similar to those in Fig 4A were performed but with antisera against H-FABP (Fig 4B) and L-FABP (Fig 4C). Immunoreactive bands of H-FABP and L-FABP were detected in heart and intestine samples, respectively, whereas these FABPs could not be detected in HUVECs and DMVECs. Moreover, no cross-reactivity with E-FABP was observed.

Cloning of E-FABP From Human Endothelial Cells

In order to determine whether E-FABP transcripts are also expressed in human endothelial cells (HUVECs and DMVECs), we performed RT-PCR analysis using primers based on the sequence that has recently been published for PA-FABP.15 Separation of the RT-PCR products by agarose gel electrophoresis and ethidium bromide staining revealed cDNA fragments of the expected sizes (a 380-bp fragment for reaction 1 and a 472-bp fragment for reactions 2 and 3; see Fig 5A). Southern blotting using the radiolabeled PA-FABP full-length cDNA as a probe further confirmed the expression of E-FABP in endothelial cells (Fig 5B). In addition, the RT-PCR products were cloned, and both strands of
H-FABP, and this may in part explain the controversy concerning the presence or the absence of FABPs in endothelial cells: several immunohistological studies have demonstrated that H-FABP is widely distributed in microvascular endothelial cells from various mammalian tissues; however, the amounts of H-FABP in endothelial cells isolated from human umbilical artery, rat heart, and bovine aorta have shown considerable variations, and in the present study, we did not find H-FABP expression in any of our endothelial cultures. Linsen et al. reported a discrepancy between high FA oxidation by isolated cardiac endothelial cells and their low content of H-FABP; they hypothesized that two FABP types have to be present in the heart (one localized in cardiomyocytes and a second in endothelial cells) or that during culturing, endothelial cells might lose their capacity to express H-FABP. However, the presence of E-FABP in cultured DMVECs was in accordance with its immunodetection in the dermal microvasculature on skin sections (authors' unpublished data, 1996). On the other hand, E-FABP could not be detected in transformed endothelial cells (HMEC-1), suggesting that this phenotype is lost during the transformation process (data not shown). We hypothesized that FABP expression in isolated endothelial cells might be conditioned by the tissue of origin and by environmental factors such as culture conditions. These data further indicate that conclusions about the in vivo content of FABPs in endothelial cells and about their putative functions should not be extrapolated exclusively from studies in vitro.

The lung was the only organ in which cell types other than endothelial cells and keratinocytes revealed E-FABP staining. Further biochemical investigation will allow us to determine whether this staining is due to the expression of E-FABP. The strongly stained Clara cells, goblet cells, and probably pneumocytes are implicated in the secretion of surfactant, mucopolysaccharides, and other components of lung fluid, respectively. Whether E-FABP might play a role in the secretory functions of these endoderm-derived cells remains to be elucidated.

The present study has shown that endothelial E-FABP is restricted to the microvasculature of various tissues, whereas larger blood vessels with muscular walls are usually immunonegative. Furthermore, not all capillaries contained E-FABP, as shown by the absence of staining in liver and kidney cortex. The heterogeneity of endothelial cells is now well established: small-vessel endothelial cells are phenotypically, biochemically, and functionally different from large-vessel endothelial cells. Variations concerning specific in vivo functions also exist between endothelial cells derived from different organs. Our results indicate that E-FABP expression in endothelial cells is related to the size of the vessels and to the nature of the tissue surrounding the endothelial cells. They indirectly suggest that E-FABP-mediated FA metabolism and/or transport occurs at the level of the microvasculature in several target organs and that E-FABP expression might also be regulated by processes implicated in cell differentiation.

Microvascular endothelial cells play a central role in the delivery of nutrients such as FA to tissues. It is generally assumed that FAs are transported from the blood compartment to the interstitium directly through the cytoplasm of endothelial cells. Several mechanisms...
probably contribute to FA transfer across the vascular barrier, but they remain poorly understood;21 the involvement of endothelial H-FABP has been hypothesized,19 but this remains controversial.21 Interestingly, E-FABP is strongly expressed in capillary endothelial cells of several tissues that participate actively in the metabolism of FA, in locations where an important traffic of free FA occurs from the blood compartment to tissue cells (and/or vice versa): around adipocytes, serving as lipid storage cells and as a readily available source of energy; in the small intestine, where intestinal FAs are absorbed; and in the microvasculature of heart and skeletal muscles, which require high levels of blood-derived FA for energy. It is noteworthy that a complex pattern of FA metabolism and transport also occurs in the placenta,25 which contains several FABP types.11,15-18,26 This organ has a high FA requirement for its own energy and structural needs and for supplying FAs to the fetus. Since fetal capillaries from placental villi strongly express E-FABP, this protein may be important for the transfer of free FAs from maternal blood to the fetus. Our data support the idea that E-FABP is involved in the uptake and transport of free FAs across the endothelial cytoplasm. The presence of E-FABP in endothelial cells of the microvasculature might also suggest that these cells must undergo FA-mediated activation to perform their functions of uptake and release of various metabolites.

Acknowledgments
This study was supported in part by the Swiss National Science Foundation (grants 32-31338.91 and 32-47057.96 to Dr Siegenthaler and grant 31-43364.95 to Dr Pepper). We are grateful to Raymonde Hotz, Kerstin Tamm, Corinne Di Sanza, and Mireille Quayzin for their skilful technical assistance.

References