Rabbit Heart Fatty Acid-Binding Protein
Isolation, Characterization, and Application of a Monoclonal Antibody
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A fatty acid-binding protein (FABP) was purified from rabbit heart and characterized with respect to size, isoelectric point, and tissue distribution. This protein was found in red muscle, diaphragm, and aorta, as well as in the heart. Amino acid composition of rabbit heart FABP differed only slightly from the human and rat proteins. Rabbit heart FABP was shown to bind two molecules of fatty acid. A monoclonal antibody was developed and used to demonstrate the feasibility of a one-step purification with affinity chromatography. Cross-reactivity was found between the human protein and the rabbit antibody, and an immunoassay was developed to human heart FABP. Levels of human heart FABP in the plasma of patients with acute myocardial infarction were significantly elevated (82±9 μg/ml) compared with patients with pulmonary edema (52±7 μg/ml; p<0.05, mean±SEM).

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Heart fatty acid-binding protein (hFABP) is one member of a multigene family of proteins characterized by their relatively small size, cytoplasmic localization, and their ability to bind hydrophobic ligands including long-chain fatty acids, retinol, retinoic acid, and several other organic anions. Distinct fatty acid-binding proteins (FABPs) have been isolated from rat liver, 1 intestine, 2 kidney, 3 adipose tissue, 4 and heart, 5,6 and the complementary DNA for rat hFABP has been obtained. 7 Unlike FABPs from other tissues, hFABP, at least in the rat, has a broad tissue distribution and is most abundant in heart and slow twitch skeletal muscle; it is present in kidney, testis, adrenal, placenta, and aortic tissue. Although the function of hFABP, like other members of this multigene family, is presumably to facilitate the uptake and intracellular metabolism of long-chain fatty acids, this function has not been rigorously established, and the possibility of pleiotropic effects for these proteins has been suggested based on effects on cell growth and differentiation. 8

In recent studies, 9 we have described the leakage of hFABP from the intact rat heart after experimentally induced ischemia and suggested that loss of this protein after ischemia may make the viable cells more vulnerable to cellular damage during reperfusion. In other studies, 10 we identified hFABP in rat aortic tissues and showed that its expression was markedly reduced in aorta, but not in other tissues, after experimental hypertension. The possibility that hFABP may have a protective role in the heart or aorta and that its reduced expression may be a causative factor in different forms of cardiovascular disease prompted us to examine the properties of this protein in the rabbit, an animal that has proven useful for studying ischemia, hypoxia, and myocardial metabolism and that is an excellent experimental model for atherosclerosis. In the present study, we have purified and characterized an hFABP from rabbit heart and produced monoclonal antibodies to this protein. One of the antibodies was used to show the feasibility of purifying the protein by affinity chromatography. In addition, monoclonal antibodies to rabbit hFABP were used to develop an enzyme-linked immunosorbent assay (ELISA) to human hFABP. With this assay we measured human hFABP and quantitated plasma levels in patients with myocardial infarction and patients with pulmonary edema.

Materials and Methods
Isolation of Rabbit Heart FABP
Rabbit hearts were purchased from Pel-Freeze Biologicals (Gilbertsville, Pennsylvania) and were...
received packed in dry ice. They were stored at -70°C until use. Hearts were minced in a 0.1 M phosphate buffer, pH 7.4, and then homogenized in 2 vol buffer with a Polytron apparatus (Brinkmann Instruments, Westbury, New York). This and all subsequent steps were carried out at 4°C. The homogenate was centrifuged at 18,600g for 20 minutes. The supernatant was then centrifuged at 150,000g in a 52.2 Ti rotor (Beckman Instruments, Fullerton, California) for 90 minutes. The resulting high speed supernatant was then applied to a Sephadex G-75 column (5 x 90 cm) preequilibrated with 30 mM Tris, pH 8.3. Elution was performed with 30 mM Tris, pH 8.3, and a low molecular weight fraction, containing predominantly myoglobin and hFABP, was obtained. This fraction was dialyzed overnight against deionized water; sodium acetate was added to the sample to a final concentration of 10 mM, pH 5.0; and the sample was quickly applied to a CM-52 column (Whatman, Clifton, New Jersey). hFABP was eluted by use of a 10-200 mM NaAc salt gradient, which effectively separated hFABP from myoglobin. Immediately after elution from the column, 1 M Tris was added to the fractions containing hFABP to neutralize the protein solution. The samples were then dialyzed overnight against 30 mM Tris, pH 8.3, and stored in aliquots at -70°C.

Protein samples were analyzed by gel electrophoresis with the method of Laemmli. Separating gels contained 15% acrylamide (National Diagnostics, Manville, New Jersey). Gels were fixed and stained in either Coomassie brilliant blue (Biorad, Richmond, California) or a silver stain (ICN Biochemicals, Costa Mesa, California). Amino acid analysis and isoelectric focusing were carried out as described previously. Tryptophan content was calculated with the equation:

\[ A = \frac{(5700 \times n \text{ Trp} + 1300 \times n \text{ Tyr})}{MW} \]

where A is the absorbance of a 1 mg/ml solution through a 1 cm path length and n is the number of amino acids (Trp and Tyr). The extinction coefficient was 1.1 for rabbit hFABP. Previously, we have confirmed the accuracy of this equation by comparing calculated and measured tryptophan content of the rat heart and kidney FABPs. Protein concentrations were measured in duplicate using the Coomassie assay (Biorad). Bovine serum albumin was used as a standard.

Liposomal-binding studies were performed with multilamellar liposomes made with egg yolk lecithin and oleic acid in a 20:1 ratio as previously described. Radiochemicals were obtained from New England Nuclear, Boston, Massachusetts.

Immunologic Methods

Six-week-old Balb/CJ male mice (Jackson Laboratory, Bar Harbor, Maine) were immunized with purified hFABP in Freund's complete adjuvant. Mice were injected intraperitoneally twice with 2 weeks between injections. Ten days after the second injection, titers were measured against hFABP, and an intravenous boost of hFABP was given to the mouse with the highest titer. Three days later, the spleen was removed, and a single cell suspension was made. After lysis of red blood cells with ammonium chloride, the donor cells were combined with SP2 myeloma cells (American Type Culture Collection), which were hypoxanthine guanine phosphoribosyl transferase negative. The cells were treated with polyethylene glycol and then gently pelleted. This pellet was then resuspended in 20% fetal calf serum (FCS) in Dulbecco's Modified Eagle Medium (DMEM) and plated the following day on microtiter plates at 1 x 10⁴/ml in hypoxanthine:aminopterine:thymidine, 20% FCS, 10% concanavalin A supernatant DMEM (high glucose) supplemented with penicillin/streptomycin, l-glutamine, and nonessential amino acids. Concanavalin A supernatant was obtained by stimulating normal spleen cells in culture with concanavalin A and then collecting the supernatant. The subsequent colonies were screened at 7-10 days with a noncompetitive ELISA to assess antibody activity. Microtiter plates were coated overnight at 4°C with 100 µl hFABP (1 µg/ml) and then washed with phosphate buffer and blocked with 0.2% bovine serum albumin. Dilutions of media from each colony were incubated in the microtiter plates. The plates were washed with phosphate buffer, and rat anti-mouse kappa immunoglobulin G linked to alkaline phosphatase was added. After incubation, the plates were developed with p-nitrophenyl phosphate and read at 410 nm with a microplate reader (model MR600, Dynatech Laboratories, Chantilly, Virginia).

The positive wells were cloned by limiting dilution in 20% FCS DMEM with peritoneal exudate cells from Balb/C mice as a feeder layer. The positive wells with one colony were selected and grown in flasks in 10% FCS DMEM. Media were collected after the cells became confluent, centrifuged to remove the cells, and then aliquoted and stored at -20°C until further use.

Isotyping. This was performed with a kit purchased from Amersham (Arlington Heights, Illinois). Ascites. One week after priming with intraperitoneal pristane (2,6,10,14-tetramethylpentadecane), four 8-week-old male Balb/CJ mice (Jackson Laboratory) were injected intraperitoneally with 10⁵ cells in 100 µl DMEM from the clone for antibody 624. Starting at 1 week, the mice were tapped every other day to remove ascitic fluid. Ascites was centrifuged at 2,300g at 4°C to remove all cells and passed over a glass wool filter to remove lipids. Further purification was carried out with an Affi-gel blue column (Biorad) by following the methods recommended by the manufacturer. Antibody was then aliquoted and stored at -70°C.

Affinity Column

By use of cyanogen bromide-linked Sepharose 4B (Pharmacia, Uppsala, Sweden), an affinity col-
um was made with antibody 624. Activation of the sepharose and binding of the antibody was done as recommended by Pharmacia. By use of purified hFABP, different conditions for application and elution were tested. At 25°C, sample was applied in 30 mM Tris buffer and was eluted with either 0.1 M glycine, pH 2.6, or with 1 M NaCl and 30 mM Tris, pH 7.8. High speed supernatant was then applied to the column; after washing with four void volumes of 100 mM NaCl plus 30 mM Tris at pH 7.8, purified FABP was eluted with either the glycine or the 1 M NaCl plus 30 mM Tris buffer.

Immunoblotting was done with the method of Burnette and a Transblot cell apparatus (Biorad). Transfer to nitrocellulose paper was done at room temperature over 2.5 hours. The transfer buffer consisted of 20 mM Tris base, 150 mM glycine, and 15% (vol/vol) methanol with a final pH of 7.4. The blot was incubated for 30 minutes with 3% gelatin to block nonspecific binding. This was followed by incubating for 30 minutes in blotting buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween) to which anti-rabbit heart FABP monoclonal antibody 624 in 1:200 dilution was added. The blot was then washed and incubated with anti-mouse immunoglobulin-linked alkaline phosphatase (Promega, Madison, Wisconsin) for 30 minutes. After washing, the blot was developed with substrate, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5-bromo-4-chloro-3-indolyl phosphate in (mM) Tris 100, NaCl 100, and MgCl2 5, pH 9.5. Thorough washing with deionized water was used to stop the reaction.

Development of ELISA

By use of one of the rabbit monoclonals (624) a competitive ELISA for human hFABP was developed with the approach described by Engvall. Initially, human hFABP was used for the standard curves, but because this is in short supply, a comparison of rabbit/rabbit standard curve competition with human/rabbit was done. A correction factor was calculated by using the curves generated, and rabbit hFABP was used for all standard curves. The same technique was used for both the human hFABP assay and for the rabbit hFABP assay. Briefly, microtiter plates (Flow Laboratories, McLean, Virginia) were coated overnight with 0.5 μg/ml hFABP in 0.1 M bicarbonate buffer, pH 9.6. Plates were washed with phosphate-buffered saline (PBS). Serial dilutions from 500 to 1 ng/well hFABP were done in triplicate for a standard curve. Samples were done in duplicate at two different dilutions. All dilutions were done with PBS. Antibody 624 was diluted 1:1,000 to 1:50 depending on its concentration and added in 100-μl aliquots to each well. After a 2-hour incubation with gentle shaking, the plates were washed with PBS and then incubated with a anti-mouse immunoglobulin-linked horseradish peroxidase (Amersham) for 1 hour. The plates were again washed with PBS followed by deionized water and then incubated with hydrogen peroxide in a phosphate and citrate buffer with o-phenylenediamine. After development for 5–15 minutes, the reaction was stopped with 4N H2SO4. Plates were read at 490 nm with a microplate reader (Dynatech Laboratories).

Human Plasma Samples

Venous blood samples were obtained from patients in the coronary care unit at Boston City Hospital within 24 hours of admission after obtaining informed consent. Samples were drawn from patients admitted with clearcut acute myocardial infarction or anginal syndrome, from patients with pulmonary edema but without evidence of myocardial ischemia, and from normal volunteers. Myocardial infarction was defined as S-T segment elevation of at least 1 mm in at least two leads and a typical presentation of chest pain. All these patients showed evolution of electrocardiographic changes and had elevation of creatinine kinase levels with positive MB fractions. Anginal syndrome was defined as clearcut myocardial ischemia as evidenced by S-T segment changes or T-wave inversions with clinical symptoms characteristic of myocardial ischemia and was distinguished from myocardial infarction by absence of evolution of electrocardiogram and normal serum creatinine kinase levels as determined by the hospital laboratory. Pulmonary edema was defined as severe congestive heart failure with rales to the apexes. Many of these patients required transient intubation until diuresis improved their oxygenation. Patients with both pulmonary edema and clear-cut myocardial infarction or anginal syndrome were included in the myocardial infarction/anginal syndrome group. Samples were centrifuged at 2,300g for 5 minutes at 4°C, aliquoted, and stored at -70°C until assay. The protocol was approved by the Human Investigation Committee at Boston City Hospital.

Statistics were performed by an analysis of variance to measure differences between groups, and the Bonferroni t test was used to test for significance. Values are expressed as mean±SEM.

Rabbit tissues used for tissue localization studies were the gift of Dr. Carl S. Apstein, Boston University School of Medicine. Human hFABP was the gift of Dr. Robert Troxler, Boston University School of Medicine. All chemicals were at least reagent grade and were obtained from Sigma (St. Louis, Missouri) unless otherwise indicated.

Results

Rabbit hFABP was purified with minor modifications of the strategy used to purify hFABP from rat and human hearts. A low molecular weight fraction containing predominantly myoglobin and hFABP was derived by gel filtration of the soluble fraction obtained by high speed ultracentrifugation of the homogenate. This fraction was further purified by cation exchange chromatography with CM-52, which selectively absorbed rabbit myoglobin and permit-
Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%) illustrating purification steps for rabbit heart fatty acid-binding protein. Lane A: High speed supernatant. Lane B: Low molecular weight fraction from G-75 column. Lane C: Pure heart fatty acid-binding protein eluted from CM-52 column.

A comparison of the isoelectric points of rat, human, and rabbit heart fatty acid-binding proteins is shown in Figure 2. The pl for rabbit hFABP was virtually identical to that of the rat protein, whereas human hFABP was slightly more acidic than the FABP from the rodent species. Table 1 shows the amino acid composition of rabbit hFABP compared with human and rat hFABPs and indicates the overall similarity between the different proteins. Rabbit hFABP is distinguished only by the presence of two prolines but was similar with respect to the absence of cysteine, relative abundance of glutamine and lysine, and the presence of two tryptophans. Although threonine was abundant, it was less so than in the rat and human proteins. In contrast, the valine content was similar in the rabbit and human protein, but greater in the rat. All three proteins contain a large amount of leucine; the rabbit hFABP has somewhat more than the rat and less than the human.

The binding characteristics of rabbit hFABP were tested by an assay using multilamellar liposomes as a source of labeled oleic acid (Figure 3). Binding was maximum at a molar ratio of 1.5:1 (oleate to protein), consistent with the previously established finding of two binding sites per mole of protein for rat and human hFABPs under similar experimental conditions.

Monoclonal antibodies were developed by use of the purified rabbit heart protein (see "Materials and Methods"). Two antibodies were obtained; the more

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Values for rat were determined by DNA sequence. Human values were based on amino acid sequence.
sensitive was produced in larger amounts by the ascites technique and used for further studies. This antibody was shown by isotyping to be an immunoglobulin G2a antibody. The specificity of this antibody was tested with hFABP from several species (Figure 4). The antibody showed a high affinity for rabbit hFABP but did not react with either rat hFABP or with myoglobin. However, cross-reactivity toward human hFABP was found, and although the affinity was less than for the rabbit protein, it was sufficient for the development of an ELISA for human hFABP.

The monoclonal antibody was used in Western blot analysis to show the tissue distribution of rabbit hFABP in the rabbit. Figure 5I shows that soluble fractions from heart, diaphragm, soleus, and gastrocnemius muscles all contained hFABP. In Figure 5III, a separate Western blot analysis showed the presence of hFABP in the aorta. The higher molecular weight bands noted on this blot are a result of nonspecific binding by the second antibody and were noted on an identical blot that was developed with second antibody but without first antibody. No hFABP was detected in brain, liver, or lung. The above findings were similar to those made for the tissue distribution of rat hFABP.

The usefulness of the monoclonal antibody was established by additional approaches. An immunoaffinity column was prepared and tested for its utility in effecting a one-step purification of rabbit hFABP. The high-speed supernatant fraction was applied directly to the column, and absorbed proteins were selectively eluted with various salt solutions. Figure 6 shows the supernatant fraction and the material that flowed directly through the column (lanes A and B); this figure indicates that most of the proteins were not retained. When the column was eluted with 0.1 M glycine, pH 2.6, a single band corresponding in size to hFABP was eluted from the column (lane C). The bands seen at the top of the gel reflect an artifact derived from the silver stain and were found even when conventionally purified hFABP was run on the gel.

Because of the cross-reactivity of antibody 624 with the human protein, we were able to measure circulating levels of hFABP in patients with acute myocardial infarction or anginal syndrome or pulmonary edema as well as in normal volunteers. The volunteers were housestaff with a mean age of 27.9±0.6 years (n=8, four males). Their mean level was 28±5 pg/ml plasma. Levels were measured in 14 patients admitted with myocardial infarction or anginal syndrome. This group had a mean age of 61.3±3.5 years, and 57% of the patients were male. The nine patients with pulmonary edema (without

**Figure 3.** Graph showing liposomal binding assay for rabbit heart fatty acid-binding protein (FABP) demonstrating maximal uptake of 1.5 mol oleic acid/mol heart FABP.

**Figure 4.** Graph showing enzyme-linked immunosorbent assay with antibody 624. Separate curves compare activity of the antibody with rabbit heart fatty acid-binding protein (hFABP), rat hFABP, and human hFABP. The antibody showed no activity against rat hFABP.
myocardial infarction or anginal syndrome) had a mean age of 65.2±2.6 years, and 56% of these patients were male. The patients with acute myocardial infarction or anginal syndrome had significantly higher levels with a mean of 83±9 μg/ml plasma compared with normals and with those with pulmonary edema, who had a level of 53±7 μg/ml. These findings are illustrated in Figure 7. Although patients with pulmonary edema had a higher mean level than normal volunteers, this difference was not statistically significant.

Discussion

These studies document several properties of rabbit hFABP and illustrate a potentially useful clinical application of a monoclonal antibody against rabbit hFABP for measuring circulating levels of the homologous protein in man. Purification of the protein was straightforward but differed somewhat from the approach used to isolate rat hFABP in that cation exchange chromatography was required to separate myoglobin from hFABP. These proteins could not be resolved adequately by anion exchange chromatography although several different experimental conditions involving pH and salt gradients were attempted. We minimized the time the protein was exposed to acidic conditions during purification since we had noticed irreversible aggregation of the liver FABP when exposed to pH conditions below 5.13 The protein was obtained in relatively high yields of about 1 mg protein per gram of tissue; these high yields indicate that the protein is abundant in rabbit heart as in rat heart.6

The physical and chemical properties of the rabbit protein were similar to that of rat and human hFABP, having an acidic isoelectric point, a molecular weight of about 14,000, and the capacity to bind more than one mole of long-chain fatty acid per mole of protein. A monoclonal antibody directed against the rabbit hFABP cross-reacted with the human, but not the rat, protein; thus, a species difference was indicated between rat and rabbit in at least one epitope. We used this monoclonal antibody 1) to establish tissue distribution of the protein, 2) to show the feasibility of purification by affinity chromatography, and 3) as a tool for measurement of the human hFABP in plasma from patients. Interestingly, there have been no previous reports on the production or use of monoclonal antibodies directed against any of the other FABPs.

The precise function(s) of hFABP remains unclear. The several FABPs show tissue selective expression as observed by us in the rabbit and has been previously been reported in the rat; hFABP is primarily found in tissues of the heart, aorta, skeletal muscle, and diaphragm with a predilection for...
HUMAN PLASMA LEVELS OF hFABP

FIGURE 7. Plot showing human heart fatty acid-binding protein (FABP) plasma levels. C, controls with no acute cardiovascular disease (○); MI, patients with myocardial infarction (●) or anginal syndrome (□) (mean value is for entire group); PE, patients with pulmonary edema (△) and pulmonary edema with known coronary artery disease (▶) (mean value is for entire group). Overlap of symbols occurs secondary to duplicate values. Each of the top three solid triangles represents two patients. * Mean ± SEM. ** p < 0.05.

Because all levels were single samples drawn within 24 hours of admission, they do not necessarily represent peak levels. Patients with pulmonary edema and no evidence by EKG or enzymes of myocardial ischemia showed a mixture of plasma levels most likely representing the mixed etiologies of their underlying cardiovascular disease. Some of these patients with known coronary artery disease (as shown in Figure 7) had no evidence of acute ischemia by EKG on presentation and had normal creatine kinase levels. The unique properties of hFABP and the deleterious effects of long-chain fatty acids raise the possibility that loss of hFABP contributes to the myocardial damage from ischemia rather than just reflecting tissue necrosis. Further work needs to be done to determine the precise function of this protein in the myocardium in the experimental animal model and in humans.

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References


KEY WORDS • myocardial metabolism • myocardial ischemia • fatty acid-binding protein
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