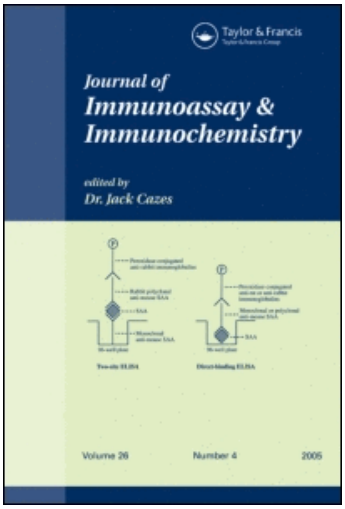


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# Establishment and Characterization of Monoclonal and Polyclonal Antibodies Against Human Intestinal Fatty Acid-Binding Protein (I-FABP) using Synthetic Regional Peptides and Recombinant I-FABP

**Satoshi Kajiura,[1] Tetsuya Yashiki,[1] Hiroyuki Funaoka,[1] Yasuhiko Ohkaru,[1] Ken Nishikura,[2] Tatsuo Kanda,[3] Yoichi Ajioka,[2] Michihiro Igarashi,[4] Katsuyoshi Hatakeyama,[3] and Hiroshi Fujii[4]**

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**Abstract:** We have succeeded in raising highly specific anti-human intestinal fatty acid-binding protein (I-FABP) monoclonal antibodies by immunizing animals with three synthetic regional peptides, i.e., the amino terminal (RP-1: N-acetylated 1-19-cysteine), middle portion (RP-2: cysteinyl-91-107) and carboxylic terminal (RP-3: cysteinyl-121-131) regions of human I-FABP, and the whole I-FABP molecule as antigens. We also raised a polyclonal antibody by immunizing with a recombinant (r) I-FABP.

To ascertain the specificity of these antibodies for human I-FABP, the immunological reactivity of each was examined by a binding assay using rI-FABP, partially purified native I-FABP and related proteins such as liver-type (L)-FABP, heart-type (H)-FABP, as well as the regional peptides as reactants, and by Western blot analysis. In addition, the expression and distribution of I-FABP in the human gastrointestinal tract were investigated by an immunohistochemical technique using a

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carboxylic terminal region-specific monoclonal antibody, 8F9, and a polyclonal antibody, DN-R2.

Our results indicated that both the monoclonal and polyclonal antibodies established in this study were highly specific for I-FABP, but not for L-FABP and H-FABP. Especially, the monoclonal antibodies raised against the regional peptides, showed regional specificity for the I-FABP molecule.

Immunoreactivity of I-FABP was demonstrated in the mucosal epithelium of the jejunum and ileum by immunohistochemical staining, and the immunoreactivity was based on the presence of the whole I-FABP molecule but not the presence of any precursors or degradation products containing a carboxylic terminal fragment.

It is concluded that some of these monoclonal and polyclonal antibodies, such as 8F9, 4205, and DN-R2, will be suitable for use in research on the immunochemistry and clinical chemistry of I-FABP because those antibodies can recognize both types of native and denatured I-FABP. In order to detect I-FABP in blood samples, it is essential to use this type of antibody, reactive to native type of I-FABP. It is anticipated that, in the near future, such a method for measuring I-FABP will be developed as a useful tool for diagnosing intestinal ischemia by using some of these antibodies.

**Keywords:** Characterization, Establishment, Immunohistochemistry, Intestinal fatty acid-binding protein (I-FABP), Monoclonal antibody, Polyclonal antibody, Synthetic regional peptide



## INTRODUCTION

Fatty acid-binding protein (FABP) is a soluble protein with a low molecular mass, i.e., 14–15 kDa, that is abundantly present in the cytoplasm of cells utilizing fatty acids as their energy source. Nine types of FABP have been reported to date, including FABPs expressed specifically in tissues such as the intestine,[1–4] liver,[5,6] heart,[7–9] brain,[10–12] adipose tissue,[13,14] epidermis,[15–17] testis,[18] myelin,[19] ileum.[20–22] The FABPs play important roles in the transportation and metabolism of long-chain fatty acids in the cells of these tissues. Although the amino acid sequences of these FABPs are conserved with high homology to each other,[23] these proteins are immunologically distinct.[24]

Intestinal-type FABP (I-FABP) is an extremely abundant, i.e., 2–3%, cytoplasmic protein localized in epithelial cells of the mucosal layer of the small intestine[25] and is part of a multigene family of low molecular weight proteins (approximately 15 kDa).

When the intestine is in ischemia due to poor mesenteric blood flow, epithelial cells in the mucosal layer of the small bowel tissue are injured and damaged due to necrosis. Under those conditions, I-FABP is thought to be easily released from the epithelial cells into the circulation. Therefore, I-FABP might be useful as a biochemical marker for early detection of intestinal ischemia. Some reports have already shown that the serum I-FABP concentrations determined by immunoassay using a polyclonal antibody to

I-FABP reflect the state of ischemia and necrosis,[26–30] as well as allograft rejection,[31–33] in the small intestine. In addition, the immunological distribution of I-FABP has been reported by several researchers using a polyclonal antibody in rat tissues.[34–36] However, to the best of our knowledge, there have been no reports of establishment of region-specific monoclonal antibodies to human I-FABP which are highly specific and usable for immunoassay and immunohistochemistry.

In the present study, we describe the establishment and characterization of monoclonal and polyclonal antibodies using three synthetic regional peptides of I-FABP and/or recombinant human I-FABP expressed as antigens in a baculovirus. The purposes of this study were to establish region-specific antibodies to I-FABP and to ascertain the specificity of the antibodies by investigating their reactivity with the related FABPs and the distribution of I-FABP immunoreactivity in the human gastrointestinal tract and related tissues using an immunohistochemical technique.



## EXPERIMENTAL

### Materials

#### Synthetic Peptides

Three regional peptides with high hydrophilicity corresponding to the amino terminal (1–19), middle portion (91–107) and carboxylic terminal (121–131) of human I-FABP (1–131) were synthesized by a conventional liquid layer peptide synthesis and purified by reverse-phase high pressure liquid chromatography with gradient elution using acetonitrile/trifluoroacetic acid (0.01–1%) as the mobile phase. A cysteine residue was introduced to the carboxylic terminal of the amino terminal peptide, the amino terminal of the middle portion and the carboxylic terminal peptide to enable a coupling reaction with a carrier protein. The amino acid sequences of these three peptides were N-acetylated-AFDSTWKVDRSENYDKFME-C (regional peptide 1: RP-1), C-GKFKRTDNGNELNTVRE (regional peptide 2: RP-2) and C-GVEAKRIFKKD (regional peptide 3: RP-3), respectively. The sulfhydryl group of the cysteine residue at the amino or carboxylic terminal of each peptide was used for conjugation with the amino groups of bovine serum albumin (BSA) when it was used for immunization.

#### Recombinant Human FABPs

Recombinant human I-FABP (rI-FABP) was expressed in Sf9 cells using the Bac-to-Bac Baculovirus Expression System (Gibco BRL, Grand Island, NY, USA).[37] Recombinant human heart-type (H)-FABP and liver-type (L)-

FABP were purchased from Institute of Chemistry and Biochemical Sensor Research (Munster, Germany). Native rat I-FABP was purified from rat small bowel tissue by the method described previously.[38]

### Human Tissues of Digestive Tract and Related Organs

Normal human tissues from the gastrointestinal tract such as the esophagus, stomach, duodenum, jejunum, ileum, colon and rectum, and other tissues including the liver, gallbladder, and pancreas were obtained from surgical specimens under informed consent from each patient, based on the regulations of the internal review board of Niigata University Medical and Dental Hospital when a surgical operation on the gastrointestinal tract was performed. All tissues were fixed in 10% formalin immediately after they were obtained and embedded in paraffin by the conventional method.



## Methods

### Purification of Native I-FABP

Preparations of human small intestinal mucosa were purchased from KAC (Kyoto, Japan) with a certificate of negativity for such viruses as hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) Human intestinal mucosa weighing approximately 100 g (wet) was homogenized in 40 mM phosphate buffer, pH 6.9, containing 150 mM sodium chloride, 1 mM ethylene diamine tetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride and 0.1% sodium azide. The homogenate was centrifuged at 38,000 × g for 60 min at 4°C. The supernatant was dialyzed against 30 mM acetate buffer, pH 5.0, and applied to a CM-32 ion-exchange column (1 cm × 7.5 cm) equilibrated with 30 mM acetate buffer, pH 5.0. After washing the column with the buffer for dialysis, elution of I-FABP from the column was performed with a linear gradient of 0–400 mM sodium chloride in acetate buffer, pH 5.0. The eluted fractions were monitored for absorbance at 280 nm and reactivity with anti-I-FABP monoclonal and/or polyclonal antibody. The fractions eluted with 90–190 mM sodium chloride were pooled. The pooled eluate was dialyzed against 30 mM borate buffer, pH 9.0, overnight at 4°C. After the dialysis, the solution was applied to a DE-52 ion-exchange column (1 cm × 7.5 cm) equilibrated with 30 mM borate buffer, pH 9.0. The passed fractions containing I-FABP immunoreactivity were collected and pooled. The column chromatography was carried out at 4°C. The purity of the native I-FABP was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and non-reduced conditions.

## Preparation of Monoclonal Antibody Against the Regional Peptides and I-FABP

The coupling reactions of the regional peptides with BSA were carried out using an Imject® Maleimide Activated Immunogen Conjugation kit (PIERCE, Rockford, IL, USA). The coupling process was based on the manufacturer's manual. In this coupling reaction the sulfhydryl group of the cysteine residue introduced to each peptide was used for conjugation with an activated amino group of BSA. In brief, each synthetic peptide (2 mg) was dissolved in 200 μL of the conjugation buffer included in the kit. Then, maleimide-activated BSA (2 mg) was dissolved in 200 μL of distilled water and added to the each peptide solution. The reaction mixture was gently agitated in an immunorotator for 2 h at room temperature. After the reaction, the reaction mixture was applied to a D-Salt Dextran Desalting Column equilibrated with the purification buffer of the kit at a flow rate of 200 μL/min to remove any excess synthetic peptide. The synthetic peptide coupled with BSA was eluted with the elution buffer of the kit, and it was used as an immunogen for preparing a monoclonal antibody specific for the regional portion of the I-FABP molecule.

Animals' care in this study was in accordance with institutional guidance. For the first immunization, 50 μg of the peptide conjugated with BSA (280 μL) was emulsified with 280 μL of Titermax classic adjuvant (Sigma, St. Louis, MO, USA) by ultrasonication under ice-cold conditions. The emulsion (0.5 mL) was injected to five sites in the back and abdomin of female BALB/c mice (4 weeks old, Charles River Japan Inc., Yokohama, Japan) subcutaneously and intracutaneously. After a 2-week interval, half content of the peptide-BSA in the same volume of the emulsion was used for the second immunization. Ten days after the second immunization, peptide-BSA (25 μg as peptide) in 500 μL of saline was injected intraperitoneally as a booster. Three days after the booster immunization, splenocytes obtained from the immunized animal were fused with mouse myeloma cells, X-63Ag8-6.5.3. (Dainippon Sumitomo Pharma, Osaka, Japan) in the presence of 50 w/v% polyethylene glycol (PEG) 4000 (Merck, Darmstadt, Germany) to establish hybridomas.

Hybridomas were selected in HAT medium and screened by checking the binding activity of the antibody secreted in the HAT medium for human recombinant (r) I-FABP, the three regional peptides conjugated with BSA or BSA immobilized in microtiter wells. In addition, some of hybridomas were examined for their reactivity with immobilized nI-FABP on nitrocellulose membrane. Hybridomas producing an anti-human I-FABP monoclonal antibody were cloned twice by limiting dilution to make them monoclonal. The monoclonal antibody-secreting hybridoma was additionally enriched in ascetic fluid from BALB/c mice pretreated by intraperitoneal injection of 1.0 mL of Pristane (Aldrich, Milwaukee, WI, USA). The immunoglobulin in the ascetic fluid was purified by ammonium sulfate fractionation and

DEAE-cellulose column chromatography. The purified immunoglobulin was stored at −20°C until use. The immunoglobulin subclass of each monoclonal antibody was determined with a mouse monoclonal antibody isotyping kit (Dainippon Sumitomo Pharma, Osaka, Japan). Monoclonal antibody against I-FABP was raised by immunizing female BALB/c mice with 50 μg of human rI-FABP and screened by the same method as described above.

## Preparation of Polyclonal Antibodies Against Human rI-FABP

Human rI-FABP (100 μg) was added to 2.0 mL of saline and then mixed with 2.0 mL of Adjuvant Complete Freund (DIFCO Laboratory, Detroit, Michigan, USA) to prepare an emulsion for immunization. Immunization was carried out 7 times biweekly by subcutaneously injecting 1.0 mL (total) of the emulsion to five sites on the back of a male New Zealand White rabbit (11 weeks old, Nippon SLC, Shizuoka, Japan). From the second immunization to the sixth immunization, half the amount of rI-FABP (50 μg) was used in the same volume of emulsion. For the final immunization 50 μg of rI-FABP in 0.5 mL of saline was used for the subcutaneous injection as a booster shot. The titer of anti-I-FABP antibody in blood drawn from the immunized animal was determined by checking the binding activity of the antibody to human rI-FABP and/or purified nI-FABP immobilized in microtiter wells. Seven days after the final immunization the whole blood of the immunized animal was collected by cannulation of the carotid artery under anesthesia. The whole blood was held at room temperature for 2 h to allow it to coagulate, and it was then centrifuged for 20 min at 1500 × g at 4°C to separate the antiserum fraction. The antiserum was subjected to affinity chromatography using a protein A Sepharose column (Amersham Biosciences, Uppsala, Sweden) to purify the rabbit polyclonal anti-human I-FABP antibody. The purified polyclonal antibody was stored frozen at −20°C until use.

## Immobilization of rI-FABP, Synthetic Regional Peptide-BSA, or BSA in Microtiter Wells

The purified rI-FABP was immobilized in microtiter wells (Labsystems, Helsinki, Finland) by physical adsorption. In brief, 100 μL of the purified rI-FABP (2.0 μg/mL of 100 mM phosphate buffer, pH 7.0) was dispensed into each microtiter well and kept for 48 h at 10°C. Then the content of each well was discarded, and the well was washed three times with 10 mM phosphate buffer containing 100 mM sodium chloride and 0.4% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) (washing buffer) to remove unbound rI-FABP molecules. Next, 300 μL of 1.0 w/v% Block Ace solution was dispensed into the well and kept for 2 h at 25°C to block unused sites in the well. The blocking media was removed just before use.

Immobilization of BSA and each of the three synthetic regional peptides conjugated with BSA was performed in the same manner, using 1.0 μg of the each peptide-BSA (as peptide) or BSA in 100 mM phosphate buffer, pH 7.0.

### Assay Procedure for Checking the Binding Activity to Immobilized rI-FABP

The diluted supernatant (1:4) of a hybridoma, diluted mouse antiserum (1:500–1:4,000) or diluted rabbit antiserum (1:25,000–1:250,000), in a volume of 100 μL, was added to wells coated with rI-FABP and held at 25°C for 60 min to react the antibody with the I-FABP. Then the content of each microtiter well was discarded, and the well was washed three times with the washing buffer to remove unreacted components in the medium or antiserum. Next, horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody (Dainippon Sumitomo Pharma, Osaka, Japan) or anti-rabbit IgG antibody (Dainippon Sumitomo Pharma, Osaka, Japan) (100 μL) was added to each microtiter well, followed by incubation for 60 min at 25°C. The content of each well was discarded and the wells were washed three times with the washing buffer to remove unreacted HRP-conjugated anti-mouse IgG or anti-rabbit IgG antibody. Then the HRP enzyme assay was started by addition of 100 μL of a color-developing reagent solution containing tetramethyl bentidine (TMB+, DAKO, Carpinteria, USA) and hydrogen peroxide to each well. After incubation for 30 min at 25°C, the reaction was terminated by addition of 100 μL of 1.6 N sulfuric acid. The absorbance of each well at 450 nm as the main wave length and 620 nm as the reference wave length was measured within 20 min using a Multiskan Biochromatic plate reader (Labsystems, Helsinki, Finland) equipped with Delta Soft II (BioMetallics Inc., Princeton, NJ, USA). As a reference procedure for screening for monoclonal antibodies using the supernatants of hybridomas, wells coated with the each synthetic peptide conjugated with BSA or BSA were used instead of wells coated with rI-FABP.

### Immunohistochemistry

Paraffin-embedded specimens of the human gastrointestinal tract, such as the esophagus, stomach, duodenum, jejunum, ileum, colon, and rectum, and related tissues including the liver, gallbladder, and pancreas after fixation in formalin were subjected to immunohistochemical study using the anti-human I-FABP monoclonal and polyclonal antibodies. Sections of each specimen were reacted with the monoclonal and polyclonal antibodies, and localization of I-FABP immunoreactivity was ascertained by visualizing with the streptavidin-biotin immunoperoxidase method. In brief, sections of the specimens were deparaffinized with alcohol and autoclaved to enhance their reactivity with the antibodies. Subsequently, the sections were treated with 0.3% hydrogen peroxide in methanol for 30 min in order to eliminate

endogenous peroxidase activity. Nonspecific binding of antiserum was blocked by incubation with 10% goat serum for 20 min at 25°C. The sections were incubated overnight at 4°C with a monoclonal antibody (1:1,000) and/or a polyclonal antibody (1:10,000). After the reaction with the antibodies, the sections were incubated with biotin-labeled goat anti-mouse IgG (Dainippon Sumitomo Pharma, Osaka, Japan) or goat anti-rabbit IgG (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min at 25°C. Then the sections were incubated with HRP-labeled streptavidin for 30 min at 25°C and were visualized by incubation with 0.01% 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals Industries, Osaka, Japan) containing 0.001% hydrogen peroxide.

Western Blotting

To ascertain the specificity of the antibodies, Western blot analysis was performed using I-FABP and related proteins such as native I-FABP partially purified from human intestinal mucosa, human rI-FABP, rH-FABP, rL-FABP and purified rat native I-FABP. The partially purified native I-FABP at 4.9 μg of the protein concentration from human intestinal mucosa and about 300 ng of human rI-FABP, rH-FABP, rL-FABP or rat native I-FABP were analyzed by polyacrylamide gel electrophoresis (PAGE) using 5–20% polyacrylamide gel (ATTO Corporation, Tokyo, Japan) in the presence of sodium dodecyl sulfate (SDS). After completion of the SDS-PAGE the separated protein bands in the gel were electrically transferred to a polyvynilidenedifluoride (PVDF) membrane for reaction with anti-I-FABP monoclonal and polyclonal antibodies. The transferred membrane was blocked with 4% Block Ace and incubated with a polyclonal antibody (10 μg/mL) or monoclonal antibody (10 μg/mL) for 60 min at room temperature. Then the membrane was washed with TTBS buffer (20 mM Tris-HCl, pH 7.4, containing 500 mM NaCl and 0.1% Tween 20) to remove the unreacted anti-I-FABP antibody. Next, the membrane was incubated with HRP-labeled anti-mouse IgG antibody solution for 60 min at room temperature and then visualized with a POD Immunostain Kit (Wako Pure Chemical Industries, Osaka, Japan).

## RESULTS

### Binding Activity of Antibody in Antiserum to the Immobilized Regional Peptides and Human rI-FABP

Table 1 presents the results for the binding activities of the antibodies in the antisera obtained from the immunized mice and rabbits with respective antigens relative to the immobilized rI-FABP and/or regional peptides. Anti-RP-1 and anti-RP-3 antisera from the mice immunized with RP-1

***Table 1.*** Binding activities of antibody in mouse and rabbit antisera for regional peptides, BSA and rI-FABP

| | | | Immobilized I-FABP, regional peptides or BSA | | | | |
|---|---|---|---|---|---|---|---|
| Antiserum | Animal | Dilution | RP-1 | RP-2 | RP-3 | BSA | rI-FABP |
| Anti-RP-1 | Mouse | 1:1,000 | >3.000 | nt | nt | 2.240 | 1.104 |
| | | 1:2,000 | >3.000 | nt | nt | 1.182 | 0.544 |
| | | 1:4,000 | >3.000 | nt | nt | 0.584 | 0.213 |
| Anti-RP-2 | Mouse | 1:1,000 | nt | >3.000 | nt | 2.904 | 0.035 |
| | | 1:2,000 | nt | >3.000 | nt | 1.683 | 0.028 |
| | | 1:4,000 | nt | 2.861 | nt | 0.846 | 0.023 |
| Anti-RP-3 | Mouse | 1:1,000 | nt | nt | >3.000 | >3.000 | >3.000 |
| | | 1:2,000 | nt | nt | >3.000 | >3.000 | >3.000 |
| | | 1:4,000 | nt | nt | >3.000 | 2.526 | 2.312 |
| Anti-rI-FABP | Mouse | 1:1,000 | nt | nt | nt | nt | >3.000 |
| | | 1:2,000 | nt | nt | nt | nt | 2.485 |
| | | 1:4,000 | nt | nt | nt | nt | 1.332 |
| Anti-rI-FABP DN-R1 | Rabbit | 1:25,000 | nt | nt | nt | nt | >3.000 |
| | | 1:125,000 | nt | nt | nt | nt | 0.966 |
| | | 1:25,0000 | nt | nt | nt | nt | 0.556 |
| Anti-rI-FABP DN-R2 | Rabbit | 1:25,000 | nt | nt | nt | nt | >3.000 |
| | | 1:12,5000 | nt | nt | nt | nt | 1.783 |
| | | 1:25,0000 | nt | nt | nt | nt | 1.190 |

The values in this table indicate the absorbance at 450 nm obtained when the binding activity to the immobilized regional peptides conjugated BSA, BSA or rI-FABP was measured using diluted antiserum. In the table, "nt" means "not tested." Each antiserum was diluted with 40 mM phosphate buffer (pH 7.0) containing 0.4% Block Ace and 150 mM sodium chloride.

(N-acetyl 1-19-C) and/or RP-3 (C-121-131) conjugated with BSA showed notable immunoreactivity with immobilized RP-1 and/or RP-3 as well as rI-FABP and BSA, respectively. In anti-RP-1 antiserum, anti-rI-FABP antibody reactive with RP-I existed together with anti-BSA antibody. In comparison with the binding activity of anti-RP-1 antiserum to rI-FABP at the dilution ratio of 1:4,000, anti-RP-3 antiserum showed more potent activity, i.e., 2.312 vs 0.213 of anti-RP-1 antiserum. The anti-RP-3 antiserum contained anti-rI-FABP antibody reactive with RP-3 and also anti-BSA antibody. On the other hand, anti-RP-2 was reactive with RP-2 and BSA, but not at all with rI-FABP. The anti-rI-FABP mouse antiserum raised against rI-FABP showed potent binding activity to rI-FABP immobilized at any dilution ratio.

Table 1 also summarizes the binding activity of the antibodies in the antisera obtained from the rabbits immunized with human rI-FABP relative to the immobilized rI-FABP. Both antisera, DN-R1 and DN-R2, showed potent reactivity with rI-FABP, but the immunoreactivity of DN-R2 was higher than that of DN-R1 at all dilutions.



## Screening of Monoclonal Anti-Human I-FABP Antibodies Derived from Mice Immunized with RP-1, RP-3 and/or rI-FABP

Eight hybridoma clones, i.e., 2B10, 10C4, 11D9, 8F9, 15B10, 17F4, 6F12 and 4H2, were selected from among the antibody-producing hybridoma cells derived from splenocytes of the mice immunized with RP-3 conjugated with BSA. They were chosen by examining the binding activity in the hybridoma cell supernatants for immobilized rI-FABP, native I-FABP, regional peptide RP-3 conjugated with BSA and BSA. The binding activities of these hybridoma clones are shown in Table 2. The 8F9 and 10C4 clones were reactive with rI-FABP and native I-FABP as well as regional peptide PR-3. The 2B10 clone was reactive only with RP-3, but not I-FABP or BSA. The 6F12, 15B10 and 17F4 clones were very similar to 2B10 in their reactivity with RP-3. On the other hand, 11D9 showed reactivity with RP-3 conjugated with BSA as well as BSA only. The clone 4H2 showed nonspecific reactivity with all materials immobilized in the wells. Among these clones, 8F9 showed the most potent binding activity to immobilized human rI-FABP, and it also showed strong immunoreactivity with nI-FABP and RP-3 conjugated with BSA, but not with immobilized BSA.

The results of the screening of hybridoma cells derived from mice immunized with RP-1 and RP-2 conjugated with BSA are shown in Tables 3 and 4, respectively. For both RP-1 and RP-2, none of the hybridoma supernatants showed potent binding activity with immobilized rI-FABP. Therefore, we did not perform any further studies except for a Western blot analysis using the clones derived using RP-1 or RP-2 conjugated with BSA as an immunogen.

***Table 2.*** Binding activity of monoclonal antibody in supernatants of hybridomas derived from splenocytes of mice immunized with RP-3

| | Immobilized I-FABP, regional peptide or BSA | | | | |
|---|---|---|---|---|---|
| Clone | rI-FABP | nI-FABP | RP-3 | BSA | Blank |
| 2B10 | 0.029 | 0.005 | >3.000 | 0.024 | 0.004 |
| 10C4 | >3.000 | 0.769 | >3.000 | 0.035 | 0.005 |
| 11D9 | 0.029 | 0.006 | >3.000 | >3.000 | 0.011 |
| 8F9 | >3.000 | 0.820 | >3.000 | 0.033 | 0.004 |
| 15B10 | 0.028 | 0.006 | >3.000 | 0.598 | 0.006 |
| 17F4 | 0.030 | 0.007 | 0.227 | 0.018 | 0.004 |
| 6F12 | 0.030 | 0.006 | >3.000 | >3.000 | 0.009 |
| 4H2 | >3.000 | 0.465 | >3.000 | >3.000 | 0.394 |

The values in this table indicate the absorbance at 450 nm obtained when the binding activity to the immobilized regional peptide RP-3 conjugated BSA, BSA, rI-FABP or native I-FABP was measured using cultured medium of these hybridoma cells. Each cultured medium of the clone was diluted with 40 mM phosphate buffer (pH 7.0). The blank assay was carried out using wells blocked with blocking solution.



On the other hand, some clones derived from the splenocytes of mice immunized with rI-FABP were selected in the same manner as described above. Table 5 summarizes the binding activity of monoclonal antibody in the hybridoma supernatants. The six clones listed in the table showed typical immunoreactivities. Three clones, 1A11, 6G3, and 4205 reacted with both rI-FABP and nI-FABP, but not the three regional peptides, RP-1, RP-2 and RP-3. Their reactivity was thus specific for I-FABP. In contrast to these three clones, 4A5 and 5E7 reacted with rI-FABP but not nI-FABP. On the other hand, the clone 6F8 reacted with rI-FABP and RP-1.

***Table 3.*** Binding activity of monoclonal antibody in supernatants of hybridomas derived from splenocytes of mice immunized with RP-1

| | Immobilized I-FABP, regional peptide or BSA | | | | |
|---|---|---|---|---|---|
| Clone | rI-FABP | nI-FABP | RP-1 | BSA | Blank |
| 1A2 | 0.177 | 0.006 | >3.000 | 0.029 | 0.004 |
| 1A3 | 0.142 | 0.004 | 2.800 | 0.061 | 0.004 |
| 6G12 | 0.134 | 0.005 | 0.811 | 0.032 | 0.005 |

The values in this table indicate the absorbance at 450 nm obtained when the binding activity to the immobilized regional peptide RP-1 conjugated BSA, BSA, rI-FABP or native I-FABP was measured using cultured medium of these hybridoma cells. Each cultured medium of the clone was diluted with 40 mM phosphate buffer (pH 7.0). The blank assay was carried out using wells blocked with blocking solution.

***Table 4.*** Binding activity of monoclonal antibody in supernatants of hybridomas derived from splenocytes of mice immunized with RP-2

| | Immobilized I-FABP, regional peptide or BSA | | | | |
|---|---|---|---|---|---|
| Clone | rI-FABP | nI-FABP | RP-2 | BSA | Blank |
| 9C9 | 0.148 | 0.008 | 2.886 | 0.050 | 0.005 |
| 11B2 | 0.139 | 0.004 | 1.095 | 0.026 | 0.003 |

The values in this table indicate the absorbance at 450 nm obtained when the binding activity to the immobilized regional peptide RP-2 conjugated BSA, BSA, rI-FABP or native I-FABP was measured using cultured medium of these hybridoma cells. Each cultured medium of the clone was diluted with 40 mM phosphate buffer (pH 7.0). The blank assay was carried out using wells blocked with blocking solution.



***Table 5.*** Binding activity of monoclonal antibody in supernatants of hybridomas derived from splenocytes of mice immunized with rI-FABP

| | Immobilized I-FABP or regional peptide | | | | | |
|---|---|---|---|---|---|---|
| Clone | rI-FABP | nI-FABP | RP-1 | RP-2 | RP-3 | Blank |
| 1A11 | >3.000 | 1.050 | 0.023 | 0.017 | 0.018 | 0.003 |
| 4A5 | >3.000 | 0.073 | 0.031 | 0.015 | 0.019 | 0.004 |
| 5E7 | >3.000 | 0.056 | 0.041 | 0.076 | 0.041 | 0.007 |
| 6F8 | 1.283 | 0.020 | 0.375 | 0.141 | 0.056 | 0.028 |
| 6G3 | >3.000 | 0.749 | 0.016 | 0.015 | 0.016 | 0.037 |
| 4205 | 2.856 | 0.525 | 0.013 | 0.015 | 0.014 | 0.012 |

The values in this table indicate the absorbance at 450 nm obtained when the binding activity to the immobilized regional peptides conjugated BSA, BSA, rI-FABP or native I-FABP was measured using cultured medium of these hybridoma cells. Each cultured medium of the clone was diluted with 40 mM phosphate buffer (pH 7.0). The blank assay was carried out using wells blocked with blocking solution.

## Western Blot Analysis

The specificities of region-specific mouse monoclonal antibody 8F9 raised against RP-3 also recognizing I-FABP and rabbit polyclonal antibody DN-R2 raised against rI-FABP were ascertained by Western blot analysis using human rI-FABP, native I-FABP partially purified from human intestinal mucosa, rL-FABP, rH-FABP and rat native I-FABP as related proteins. The results of analysis by SDS-PAGE is shown in (Figure 1(a)), and the results of Western blot analysis using 8F9, 4205 and DN-R2 antibodies are shown in (Figures 1(b), 1(c) and 1(d)), respectively. rI-FABP showed a single band at the position of 15 kDa in the SDS-PAGE analysis. In the case of partially purified human and rat nI-FABP, some contamination with a low molecular

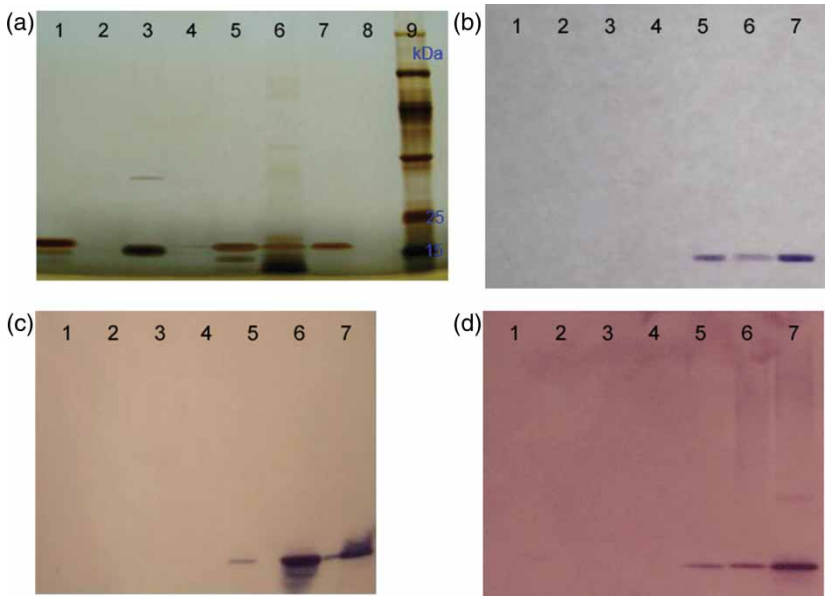


***Figure 1.*** SDS-PAGE and Western blot analysis of I-FABP and relative proteins (a) SDS-PAGE. The SDS-PAGE was carried out by using 5–20% polyacrylamide gel for analyzing proteins under reduced condition described in the text. Lane 1: human rH-FABP (347 ng/lane), lane 2: blank, lane 3: human rL-FABP (293 ng/lane), lane 4: blank, lane 5: purified rat native I-FABP (330 ng/lane), lane 6: partially purified human native I-FABP, lane 7: human rI-FABP (330 ng/lane), lane 8: blank, lane 9: molecular size marker; (b) Western blot analysis by 8F9. The Western blot analysis was carried out by using 8F9 as an anti-I-FABP monoclonal antibody described in the text. The sample of each lane was the same in order described in (a) SDS-PAGE; (c) Western blot analysis by 4205. The Western blot analysis was carried out by using 4205 as an anti-I-FABP monoclonal antibody described in the text. The sample of each lane was the same in order described in (a) SDS-PAGE; (d) Western blot analysis by DN-R2. The Western blot analysis was carried out by using DN-R2 as an anti-I-FABP polyclonal antibody described in the text. The sample of each lane was the same in order described in (a) SDS-PAGE.

weight was observed. In the Western blot analysis, both the monoclonal and polyclonal antibodies showed strong reactivity with human rI-FABP, partially purified native human and rat I-FABP at the molecular size of approximately 15 kDa but not at any smaller or larger molecular size. No reactivity was observed with other FABPs such as human rL-FABP and rH-FABP. In addition, no reactivity with other proteins present in the sample preparations, including proteins derived from the human intestinal mucosa, was detected for either 8F9, 4205 or DN-R2, as shown in (Figures 1(b), 1(c) and 1(d)). In Figures 2 (a) and (b), the reactivity of 1A2 derived from hybridomas immunized with RP-1 and 9C9 derived from hybridomas

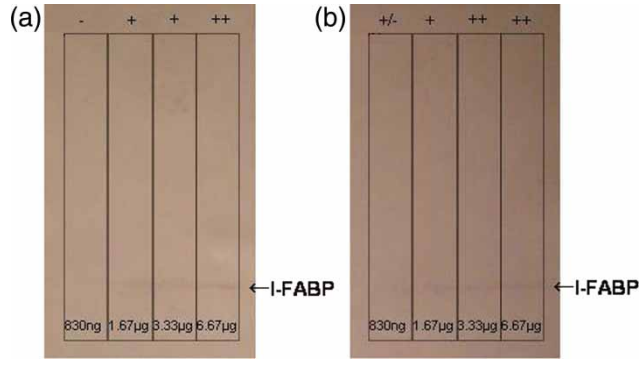


***Figure 2.*** Western blot analysis of I-FABP with 1A2 and 9C9. Western blot analysis by 1A2. The Western blot analysis was carried out by using 1A2 as described in the text. Various amount of human rI-FABP, 0.83–6.67 μg/lane, was applied and analyzed; (b) Western blot analysis by 9C9. The Western blot analysis was carried out by using 9C9 as described in the text. Various amounts of human rI-FABP, 0.83–6.67 μg/lane, was applied and analyzed.



immunized with RP-2 against various amount of rI-FABP was shown by Western blot analysis, respectively. Both monoclonal antibodies showed the reactivity to human rI-FABP dose-dependently.

## Immunohistochemistry

The specificities of mouse monoclonal antibody 8F9 and rabbit polyclonal antibody DN-R2 were ascertained by immunohistochemical studies using fixed sections of human tissues, such as the esophagus, stomach, duodenum, proximal ileum, terminal part of the ileum, jejunum, colon, rectum, liver, gallbladder and pancreas. The 8F9 and DN-R2 antibodies were used as representatives of monoclonal and polyclonal antibodies, respectively.

Intensive immunostaining by 8F9 as an anti-human I-FABP monoclonal antibody was observed in the mucosal epithelium of the proximal ileum, the terminal part of the ileum and the jejunum (Figure 3(a)), whereas weak staining was observed in the crypts of the jejunum and the proximal ileum (Figure 4(a)), the glandular cells of the duodenum, the mucosal epithelium of the colon and the rectum (Figure 5(a)) and the epithelium of the gallbladder. In addition, localized staining was observed in the wall cells and the mucosal epithelium of the antrum of the stomach, and the colon, rectum and gallbladder. On the other hand, staining was negative for the esophagus, liver (Figure 6(a)) and pancreas.

DN-R2, tested as an anti-human I-FABP polyclonal antibody, showed intensive staining in the mucosal epithelium of the jejunum (Figure 3(b))

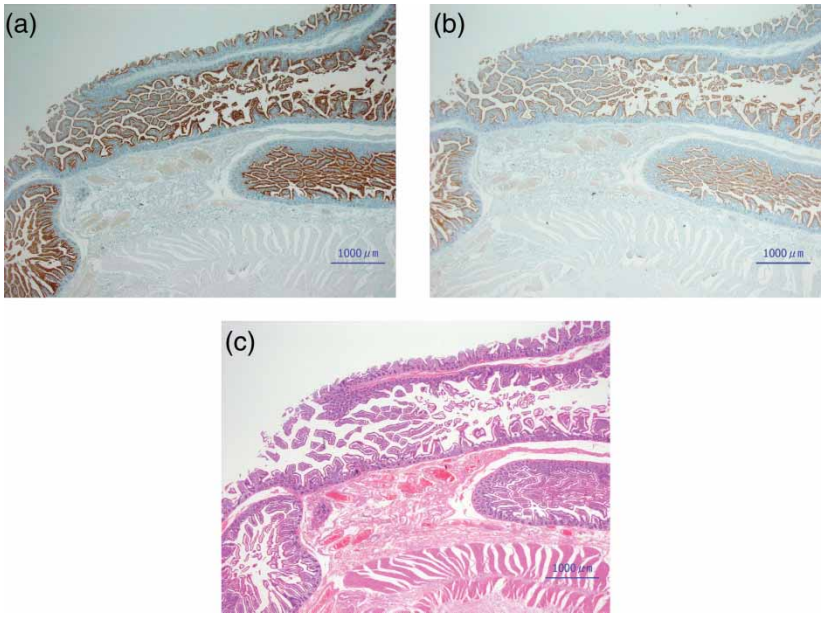


***Figure 3.*** Immunohistochemical localization of I-FABP in the jejunum. The immunoreactivity of I-FABP with anti-I-FABP antibody was ascertained by avidin-biotin complex method described in the text. (a) Immunostaining with 8F9; (b) Immunostaining with DN-R2; (c) Hematoxylin-eosin staining.



and the proximal and terminal parts of the ileum (Figure 4(b)). Weak staining was observed in the wall cells of the stomach, the mucosal epithelium of the duodenum, rectum and colon (Figure 5(b)), and the epithelium of the gallbladder. On the other hand, DN-R2 showed no reactivity in the crypts of the ileum, esophagus, liver (Figure 6(b)) or pancreas. As a reference staining to ascertain the histological and morphological state of each sample, hematoxylin-eosin staining was performed on the jejunum (Figure 3(c)), ileum (Figure 4(c)), colon (Figure 5(c)) and liver (Figure 6(c)).

The immunoreactivity of I-FABP in the gastrointestinal tract by immunohistochemical technique using anti-human I-FABP monoclonal 8F9 and polyclonal DN-R2 was recognized predominantly in the mucosal epithelium, especially in the apical portion. Moreover, the intensity of immunostaining showed a gradient distribution from strongly positive in the apical portion to weakly positive in the basolateral portion of the mucosal epithelium. On top of that, the I-FABP immunoreactivity was expressed more strongly in the jejunum than the other parts of the small intestine, and the expression in the small bowel gradually decreased as the distance from the jejunum increased.

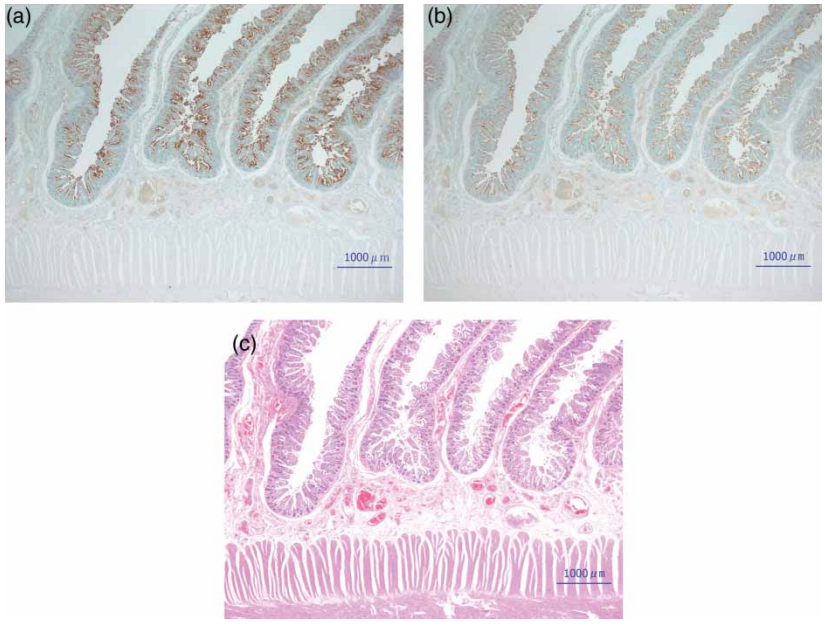


***Figure 4.*** Immunohistochemical localization of I-FABP in the ileum. The immunoreactivity of I-FABP with anti-I-FABP antibody was ascertained by the avidin-biotin complex method as described in the text. (a) Immunostaining with 8F9; (b) Immunostaining with DN-R2; (c) Hematoxylin-eosin staining.



## DISCUSSION

It is well known that I-FABP, a soluble protein, is a member of the FABP multigene family with a low molecular weight of approximately 15 kDa that is present in the cytoplasm of mucosal cells of the small bowel and functions as a transporter of long-chain fatty acids in the cells.[39] In the present study, we used three synthetic regional peptides, RP-1 (N-acetylated 1-19-C), RP-2 (C-91-107), and RP-3 (C-121-131), of human I-FABP to establish region-specific antibodies against I-FABP. We also used rI-FABP for immunization to obtain antibodies recognizing a conformational epitope of the I-FABP molecule. Polyclonal antibodies have been utilized in immunological measurements and immunohistochemical studies for the purpose of specific detection of I-FABP[33–36] but, to date, there have been no published reports on region-specific monoclonal antibodies.

In our present study, immunization with RP-1 and RP-2 conjugated with BSA did not yield any valid monoclonal antibodies. This might be due to differences in the conformational structures of the corresponding peptides in the human I-FABP molecule and those of the synthetic regional peptides conjugated with BSA. In the antisera raised against these regional peptides, the

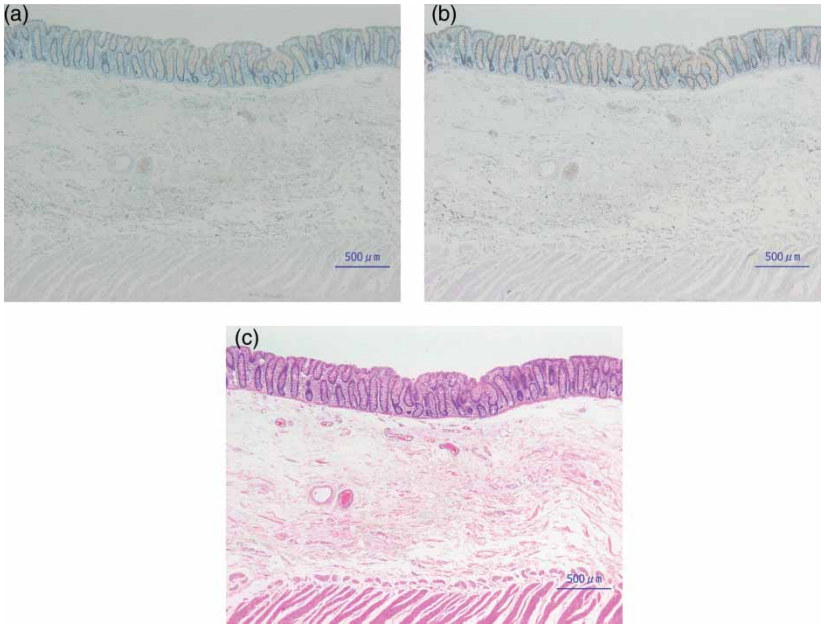


***Figure 5.*** Immunohistochemical localization of I-FABP in the colon and rectum. The immunoreactivity of I-FABP with anti-I-FABP antibody was ascertained by avidin-biotin complex method as described in the text. (a) Immunostaining with 8F9; (b) Immunostaining with DN-R2; (c) Hematoxylin-eosin staining.



reactivities of the antibodies for the immobilized rI-FABP were rather weak compared to that of the antibodies in the anti-RP-3 antiserum. On the other hand, among the hybridomas raised against RP-3, 8F9 was the most reactive monoclonal antibody for I-FABP and it was specific for the region of the carboxylic terminal of human I-FABP. Some hybridomas produced anti-RP-3 antibodies which reacted with the regional peptide RP-3 itself but not with I-FABP, and others produced anti-BSA antibodies. This indicates that anti-BSA antibody is easily raised because of BSA's large molecular size and the difference in the amino acid sequences between murine and bovine serum albumins. We surmised that monoclonal antibodies recognizing the sequential and/or conformational epitopes of the I-FABP molecule were not easily raised since the molecular structure of immobilized I-FABP in the microtiter well used in the binding assay for screening hybridoma cells might be different from the original native conformational structure in a soluble state.

When rI-FABP was used for the immunization, 1A11 and 4205 monoclonal antibodies and DN-R2 polyclonal antibody showed potent reactivity for rI-FABP as well as nI-FABP. It was thought that the epitope recognized by 1A11 and 4205 was in the region different from the amino acids

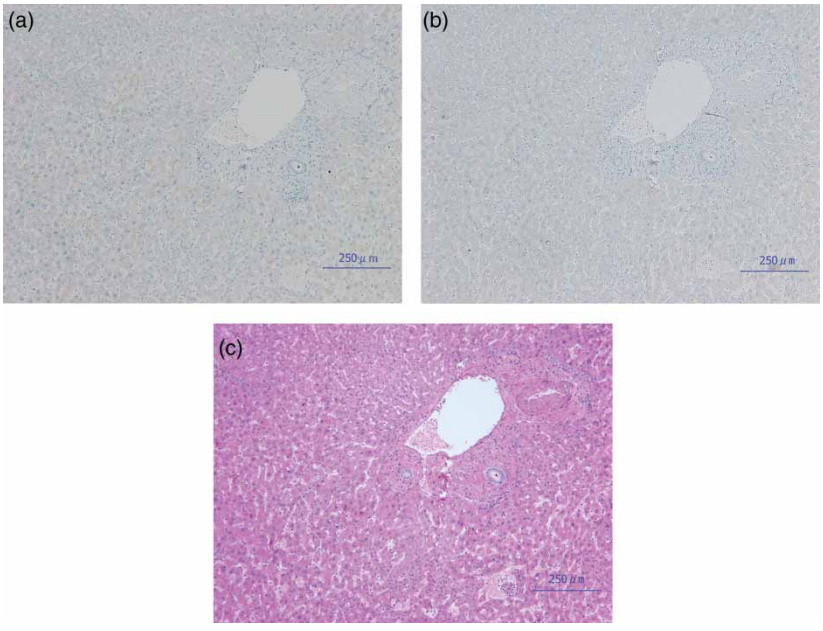


***Figure 6.*** Immunohistochemical localization of I-FABP in the liver. The immunoreactivity of I-FABP with anti-I-FABP antibody was ascertained by avidin-biotin complex method as described in the text. (a) Immunostaining with 8F9; (b) Immunostaining with DN-R2; (c) Hematoxylin-eosin staining.



sequence corresponding to RP-1, RP-2, and RP-3 present in human I-FABP molecule.

As representatives of region-specific monoclonal and polyclonal antibodies, we investigated the specificity and characteristics of 8F9 and DN-R2 antibodies by Western blot analysis, and the results showed these antibodies to be very specific to I-FABP. Monoclonal antibody 8F9, raised against RP-3, which is the carboxylic terminal fragment of human I-FABP, showed high specificity for human I-FABP and rat I-FABP. This indicates that 8F9 recognizes the shared amino acid sequence in the region of the carboxylic terminal of human and rat I-FABP, i.e., GVEAK-RIFKK. In the Western blot analysis, 8F9 reacted only with I-FABP at the position of 15 kDa, and not with any other large or small molecular weight molecules such as precursors and degradation products containing carboxylic terminal fragments of I-FABP. In addition, the antibody did not recognize FABP-related proteins such as human L-FABP and H-FABP. DN-R2 polyclonal antibody, raised against human rI-FABP, also showed high specificity for I-FABP, which indicates that DN-R2 recognizes an I-FABP specific region that includes conformational epitopes of the I-FABP molecule.

Furthermore, the specificities of the 8F9 and DN-R2 anti-I-FABP antibodies were assessed by investigating the immunohistochemical localization of I-FABP in the human gastrointestinal tract and related tissues. As reported previously,[36] I-FABP immunoreactivity was observed only in the jejunum and ileum. Staining was not observed in any other gastrointestinal tract or related tissues. Interestingly, the immunoreactivity of human I-FABP was located predominantly in the jejunum, and it gradually decreased with greater distance from the jejunum. Regarding the cellular localization of I-FABP immunoreactivity in the intestine, immunostaining was observed in the mucosal epithelium, especially in the apical region in comparison with the basal portion. These findings are almost the same as reported previously using polyclonal antibody.[34] In the papers, it was demonstrated that the staining was more intense in the proximal rather than distal intestine, in the villous rather than crypt cells, and in the apex rather than the base of intestinal cells. These results suggest that the monoclonal antibody, 8F9, and the polyclonal antibody, DN-R2, are very specific for human I-FABP. In addition, the I-FABP immunoreactivity was concentrated in the brush border of the mucosal epithelium, which means that I-FABP may play an important role in differentiation and maturation of the mucosal epithelium of the small bowel. Regarding the expression of I-FABP, it has been reported that I-FABP was expressed only in the small bowel, especially in the jejunum, duodenum and ileum.[40] The staining with these two antibodies was thought to indicate the presence of the whole I-FABP molecule but for C-terminal fragment of I-FABP based on the same staining pattern and verdict of the Western blot analysis.

Since L-FABP and I-FABP both exhibit a cephalocaudal gradient of expression in enterocytes along the duodenal-colon axis, if an anti-I-FABP antibody were to cross-react with L-FABP, it would not be possible to evaluate the results of immunostaining. However, because both the 8F9 and DN-R2 antibodies established in this study were highly specific for I-FABP, the staining indicated the existence of I-FABP. The highest expression of I-FABP was observed in the intestine, which indicates that this portion of the gastrointestinal tract may be carrying out the most active energy metabolism using fatty acids. Human I-FABP thus exhibits a distinctive, confined tissue distribution different from other FABPs and can be expected to serve as a useful cellular marker for enterocytes.

It is well known that L-FABP and H-FABP are both expressed in the small intestine as members of the FABP family, and the amino acid sequence homology between I-FABP and these FABPs is approximately 30%. Although L-FABP is expressed in large amounts in the liver, we detected no immunostaining of I-FABP in hepatic tissue. In addition, in Western blot analysis these antibodies showed immunoreactivity for native I-FABP and rI-FABP, but no reactivity for L-FABP or H-FABP. These results indicate that 8F9 and DN-R2 are highly specific for I-FABP. In this experiment we used rat I-FABP as well as human I-FABP for assessment of

the reactivity of the antibodies. These monoclonal and polyclonal antibodies also recognized rodent I-FABP. This reactivity might depend on the homology (approximately 80%) of the amino acid sequences of rat I-FABP and human I-FABP. These results suggest that the antibodies established in the present study are highly specific for human I-FABP on the basis of the fact that immunopositivity for I-FABP was observed in the mucosal epithelium, especially in the small bowel tissue.

It is concluded that some of these monoclonal and polyclonal antibodies such as 8F9, 4205, and DN-R2 will be suitable for use in research on the immunochemistry and clinical chemistry of I-FABP because those antibodies can recognize both types of native and denatured I-FABP. In order to detect I-FABP in blood samples it is essential to use this type of antibodies reactive to native type of I-FABP. It is anticipated that in the near future such a method for measuring I-FABP will be developed as a useful tool for diagnosing intestinal ischemia by using some of these antibodies.



## ABBREVIATIONS

H-FABP, heart-type fatty acid-binding protein; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver-type fatty acid-binding protein; rI-FABP, recombinant intestinal fatty acid-binding protein. BSA; bovine serum albumin, HBV; hepatitis B virus, HCV; hepatitis C virus, HIV; human immunodeficiency virus, SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis, PEG; polyethylene glycol, HRP; horseradish peroxidase, PVDF; polyvynilidenedifluoride.

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