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# Intestinal Fatty Acid-Binding Protein as a Sensitive Marker of Intestinal Ischemia

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*Determination of the serum level of intestinal fatty acid-binding protein has been used to detect rat intestinal ischemia following ligation or 30-min occlusion of the superior mesenteric artery. The normal values were under the minimal detectable level of less than 2 ng/ml in all the 10 rats. The serum fatty acid-binding protein level increased rapidly, to 340.7 ± 54.6, 438.5 ± 40.1, 388.1 ± 37.4, and 292.2 ± 95.7 ng/ml ($P < 0.01$) at 1, 2, 4, and 8 hr after ligation, respectively. It also increased, to 347.2 ± 127.7 ng/ml ($P < 0.01$) at 1 hr, after a 30-min transient occlusion and then returned to a normal level. Histological studies showed destruction of the villi, disappearance of the mucosa, and transmural necrosis with the progress of time after ligation, while no remarkable morphological change was observed following 30-min transient occlusion. These observations strongly suggest that the intestinal fatty acid-binding protein is a useful biochemical marker for intestinal ischemia, particularly in the early reversible phase.*

**KEY WORDS:** fatty acid-binding protein; serum marker; intestinal ischemia; superior mesenteric artery; enzyme immunoassay.

Acute intestinal ischemic syndromes representing superior mesenteric arterial occlusion require a rapid reliable diagnosis and surgical intervention in the early stages of an attack (1). Their diagnosis is ultimately dependent upon physical examination, since reliable biochemical diagnostic methods are unavailable at present.

Fatty acid-binding proteins (FABPs) are known to be localized in the cytosol of mammalian tissues and to have molecular masses of 14–15 kDa (2–8). The expression of the intestinal FABP (I-FABP) among the different molecular species of FABPs is known to be restricted to enterocytes and surface mucous cells of the gastric mucosa (9, 10). This tissue specific localization, the cytosolic origin, and the relatively low molecular mass of I-FABP facilitated the investigation of its leakage into the circulation from enterocytes under intestinal ischemic condition and evaluation of I-FABP as a possible biochemical marker for acute intestinal ischemic diseases.

To examine this possibility, we have prepared a specific polyclonal antibody to this protein in rabbits and have developed an enzyme immunoassay system involving the antibody. We have applied the system to experimental models, superior mesenteric artery (SMA) ligation and 30-min SMA transient occlusion, of rat intestinal ischemia and have measured I-FABP in rat serum. Serum creatine phosphokinase (CPK) and alkaline phosphatase

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(AP), representing enzymes associated with the intestine, were also measured for comparison (11). In addition, intestinal mucosal damage was investigated histologically to evaluate the relationship with the level of serum I-FABP.

The present results strongly suggest that I-FABP in serum is a valuable biochemical marker for intestinal ischemia, being sensitive in the early, reversible phase of intestinal ischemia.

## MATERIALS AND METHODS

**Animals and Models.** Animal experiments conformed to the Guidelines for Animal Experiment of Niigata University. Male Sprague-Dawley rats weighing 300–450 g were fasted for 12 hr and then anesthetized intraperitoneally with 40 mg/kg of sodium pentobarbital. Three groups were established. The control group underwent laparotomy only. The SMA ligation group had the SMA ligated with a silk ligature and cut just distal to take off the colonic branches after laparotomy. The 30-min SMA transient occlusion group had the SMA occluded for 30 min with a vascular clamp, allowing reperfusion at the same location as the ligation. Intestinal ischemia was confirmed by the contraction and pallor of the small intestine. After the 30-min transient occlusion, the clamp was removed for the blood flow to the intestines to recover. Recirculation was confirmed by inspection. Rats that regained consciousness were reanesthetized with ether during the course of the waiting period of 1–8 hr.

Blood samples were collected from the inferior vena cava of sacrificed rats at 1, 2, 4, and 8 hr after laparotomy, and the serum was separated by centrifugation at 3000 rpm (KN-70; Kubota, Tokyo) for 10 min. Histological specimens (3 cm) were taken from the midpoint of the small intestine and fixed immediately after resecting in formalin and stained with hematoxylin and eosin (H&E). Each section was histologically examined as to the seriousness of ischemic injury.

Normal blood samples to assess serum I-FABP were obtained from the inferior vena cava immediately after laparotomy. There were no differences in the I-FABP values between the inferior vena cava and the periphery (data not shown).

**Polyclonal Antibody Against Rat I-FABP.** I-FABP was isolated and purified from the small intestines of Sprague-Dawley rats as described previously (12). Antiserum against the protein was raised in rabbits by injection of the protein emulsified with complete Freund's adjuvant (Difco, Detroit, Michigan) intradermally followed by booster doses in incomplete Freund's adjuvant at two-week intervals. In a double-immunodiffusion test, the antiserum gave a single precipitin line with rat I-FABP and rat intestinal cytosol, respectively, whereas no precipitin line was observed with human intestinal cytosol. Monospecific antibody was purified from the antiserum using an I-FABP-coupled Sepharose 6MB (0.6 mg/ml, I-FABP/wet gel) affinity column. The monospecific anti-I-FABP antibody was conjugated with horseradish peroxidase and applied to an enzyme immunoassay.

**Enzyme Immunoassay of Serum I-FABP.** The serum I-FABP level was determined by an enzyme immunoassay. The assay was performed as follows: The microtiter plates were washed twice with PBS containing 0.05% Tween 20 (washing buffer). Serum was diluted to 10-fold and 100-fold with PBS containing 0.05% Tween 20 and 0.5% BSA. Then 100 μl of a serum sample was incubated in a well for 1 hr at 37° C. After the well had been washed three times, 100 μl of the peroxidase-conjugated anti-I-FABP working solution was incubated in the well for another hour at 25° C. The well was washed three times with washing buffer and then the plate was incubated for 15 min with 100 μl of orthophenylenediamine (33 mg/ml) in 0.1 M citrate phosphate buffer, pH 4.9. The reaction was terminated with 100 μl of 2 N sulfuric acid. The absorbance at 492 nm was read with an EIA Microplate reader (Bio-Rad). The analysis of serum I-FABP level was performed within 3 hr. All samples were assayed in triplicate. A standard curve for I-FABP was obtained and appropriate blanks were run for each assay. The I-FABP concentrations of samples were read directly from the standard curve.

**Assaying of Serum Enzymes Associated with Intestines.** The activities of CPK and AP in serum were measured using commercial spectrophotometric kits (Wako Chemicals, Osaka) with creatine phosphate tetrazolium salt and phenylphosphate as substrates, respectively.

**Statistical Analysis.** The results are expressed the means ± SE for $N = 8$ determinations. Differences between groups were evaluated using Student's $t$ test, significant differences being defined as those with a $P$ value of $<0.05$.

## RESULTS

**I-FABP Assay.** To prevent interference by serum, we subjected serum samples to the assay after 10-fold dilution. Figure 1 shows representative standard curves for I-FABP with and without normal rat serum, using the enzyme immunoassay. Serum had no effect with this method. The detection limit of this method was found to be 0.2 ng/ml, ie, 2 ng/ml in serum. The assay was reproducible and sensitive in the range of 0.2–50 ng/ml. The normal serum level was lower than 2 ng/ml in all 10 serum samples.

**Effect of Ligation.** Rats in the control group (Figure 2) showed no change in the serum I-FABP level, which ranged from 2 to 10 ng/ml throughout the experiment. AP did not show any significant changes in these serum samples either, ranging from 8.2 to 9.8 K-A. Serum CPK increased gradually from 406.8 ± 50.3 to 820.4 ± 168.5 IU/liter at 4 hr and had returned to 281.1 ± 68.2 IU/liter at 8 hr. In the SMA ligation group (Figure 2), serum I-FABP increased explosively within 1 hr after ligation. It reached the peak value (438.5 ± 40.1 ng/ml) at 2 hr after ligation and then remained at

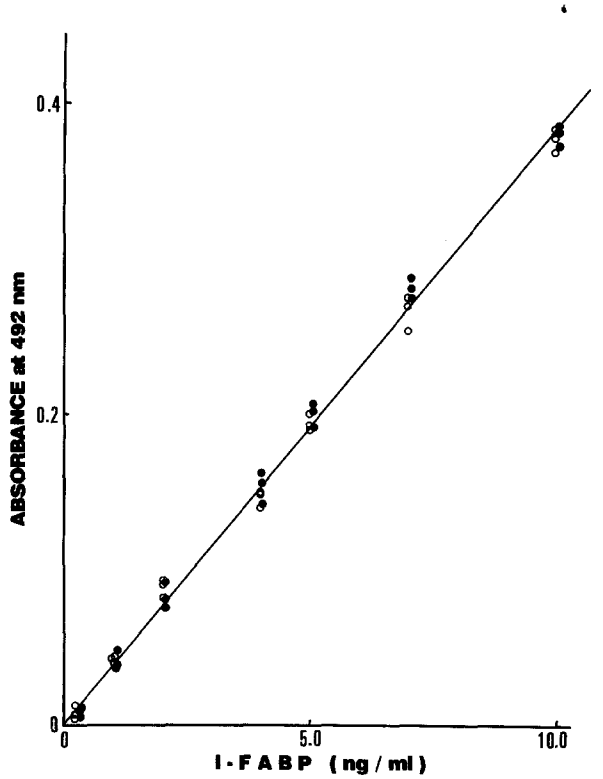


**Fig 1.** Quantitation of serum I-FABP by an enzyme immunoassay. The effect of serum on the assaying of the purified rat I-FABP was examined in the presence (●) and the absence (○) of normal serum.

292.2 ± 95.7 ng/ml for over 8 hr, with a gradual decrease. The AP value was significantly elevated at 4 and 8 hr after ligation compared with the case of a sham operation. Serum CPK did not increase until 8 hr after ligation. Thus, serum I-FABP increased sharply and earlier than AP and CPK.

**Effect of 30-min Occlusion.** Figure 3 shows the

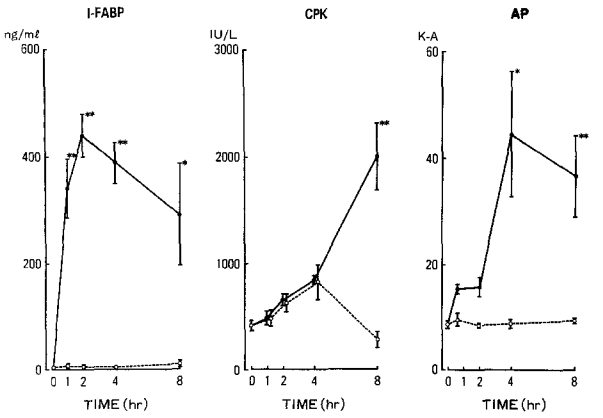


**Fig 2.** Effect of SMA ligation on the serum levels of I-FABP, CPK, and AP: the SMA ligation (●) and control rats (○). Values are means ± SE, $N = 8$. Significance of difference between treated and control rats: $*P < 0.05$, $**P < 0.01$.

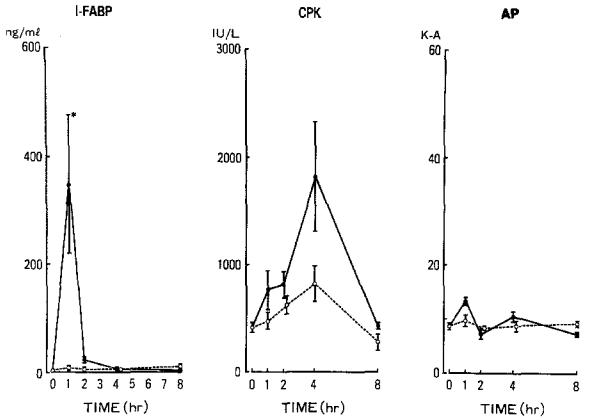


**Fig 3.** Effect of transient 30-min occlusion of the SMA on the serum I-FABP CPK and AP levels: transient 30-min SMA occlusion (●) and control (○) rats. Values are described in the legend to Figure 2.

time courses of the serum I-FABP, CPK, and AP values after 30-min transient occlusion followed by reperfusion. The serum I-FABP value at 1 hr showed significant elevation ($P < 0.05$), being on average 347.7 ± 127.7 ng/ml, although the values varied: 77, 82, 135, 164, 202, 305, 770, and 1047 ng/ml. I-FABP returned rapidly to normal within 4 hr. In contrast to I-FABP, neither CPK nor AP changed significantly throughout the experiment.

**Histological Findings.** On light microscopy, some destruction and shortening of villi were observed 1 hr after ligation of the SMA, as shown in Figure 4A. Two hours after ligation, degeneration of the intestinal epithelium and disappearance of the villi were evident (Figure 4B). As the time after ligation increased, the degeneration advanced deeper, reaching the submucosal and muscular layers of the intestinal wall (Figure 4C). The mucosa had disappeared completely and necrosis had reached the serosa at 8 hr after ligation (Figure 4D). In contrast, there was no remarkable histological change throughout the experiment involving 30-min transient occlusion of the SMA (Figure 5).

## DISCUSSION

Measurement of serum levels of organ-specific enzymes has been used to identify injured organs or to evaluate the severity of the injury, eg, GPT for liver injury or CPK for that of the myocardium. With respect to the intestines, however, no diagnostic biochemical marker has been established yet. Although an early diagnosis is essential to save a patient from acute intestinal ischemia, the lack of

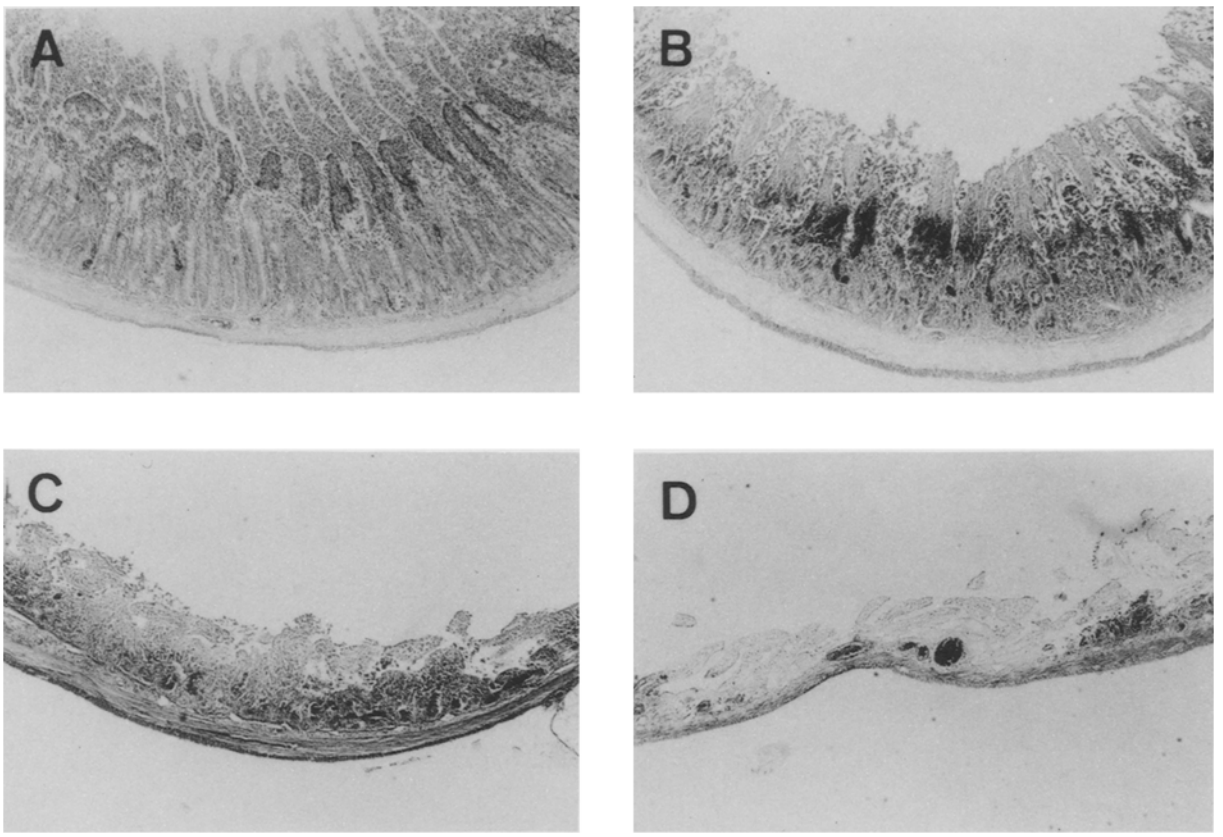


**Fig 4.** Histopathological changes of the rat intestine after SMA ligation. (A) Ischemic intestine 1 hr after SMA ligation. Shortening and focal necrosis of villi are evident (H&E; magnification ×16). (B) ischemic intestine 2 hr after SMA ligation. Mucosal hemorrhage and disappearance of the villi can be observed (H&E; magnification ×16). (C) Ischemic intestine 4 hr after SMA ligation. Transmucosal necrosis is remarkable. No crypt architecture can be observed (H&E; magnification ×16). (D) Ischemic intestine 8 hr after SMA ligation. Transmural necrosis can be observed (H&E; magnification ×16).

reliable biochemical markers has meant that ischemia remains devastating.

FABPs comprise a family of low-molecular-mass proteins (~15 kDa) that are capable of binding long-chain fatty acids (2–8). It is known that there are several types of FABPs, which exhibit organ specific expression. Expression of I-FABP is restricted to enterocytes of the small intestine and some kinds of cells in the gastric mucosa (9, 10). The unique location of I-FABP led us to investigate the possibility that the protein may be a useful serum marker for intestinal injury, especially for ischemic damage. Several enzymes have been reported to be means of detecting intestinal ischemia, including glutamate oxaloacetic transaminase (GOT), lactate dehydrogenase (LDH), AP, CPK, hexosaminidase, and diamine oxidase (DAO) (11, 13–15). None of them are localized solely in the intestine, and cumbersome isolation of isoforms may be required in some cases. Porcine ileal polypeptide (PIP) was reported as a marker for bowel ischemia (16), but it may not be applicable when the upper small intestine is involved, since it is restricted in the terminal ileum (17). I-FABP is dis-

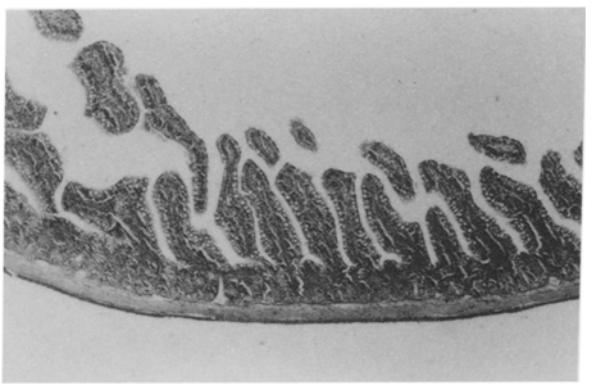

**Fig 5.** Photomicrograph of the rat intestine 1 hr after 30-min SMA occlusion. There is no remarkable change (H&E; magnification ×16).

tributed throughout the entire small intestine and is denser in the villi than in the crypts (9). The latter character may be of some advantage for the use of I-FABP as an ischemic marker for the intestine, since the mucosa is susceptible to hypoxic conditions and intestinal ischemia affects the villi first (18, 19). I-FABP has some other good properties as a biochemical marker of intestinal ischemia. First, it is a very abundant protein, accounting for 2–3% of cytosolic proteins in the intestinal epithelium (20). This abundance makes it easy to detect directly using an enzyme immunoassay, despite its lack of proper enzymatic activity. Second, I-FABP exists in the cytosol (21), suggesting that it is released easily into the circulation when the membranous permeability increases with ischemia. Third, the low molecular mass of I-FABP is suggestive of a tendency for it to leak. In addition, CPK and hexosaminidase are heat-labile enzymes requiring careful handling (22), while serum I-FABP is quite stable for 24 hr at room temperature, with a recovery of over 95%.

In this study, the blood samples are collected from the inferior vena cava, since samples from the heart or tail vein were inappropriate for measurement of CPK because of the increased activity caused by muscular crushing. The time course of the serum I-FABP level after SMA ligation revealed that I-FABP increased explosively within an hour after ligation and then remained at a high value for over 8 hr, with gradual decrease. The decrease may be attributed partly to clearance of I-FABP from the circulation, although the site and the rate of the clearance have not been examined yet. In addition, the decrease in serum I-FABP at a late stage after ligation may be explained by the mucosal exfoliation observed histologically in corresponding specimens. The same tendency was observed in the time course of the serum AP level. Although AP, as well as I-FABP, exists in the mucosa in the intestine, the change in serum AP was delayed and not so clear as that in I-FABP. The fact that AP is attached to the membrane accounts for this inferiority (23). The serum CPK level requires ischemic conditions for longer than 8 hr for elevation, in contrast to serum I-FABP and AP, reflecting its muscular origin in the intestine. Thus, the time-dependent changes in marker enzymes and their tissue origins agreed well with the histological findings (Figure 4).

In the 30-min-occlusion group, only the serum I-FABP value at 1 hr was elevated, although it varied widely compared with that in the ligation group (Figure 3). The occlusion time of 30 min may be very critical for reversible damage to the intestinal mucosa (24). It should be emphasized that the elevation of the serum I-FABP level was observed before pathologic microscopic findings appeared in the intestinal tissues, particularly in the early reversible stage of ischemic damage. This is a very important clinical diagnostic feature. If these observations can be extended to humans, we could diagnose acute intestinal ischemia before the onset of gangrene and timely intervention could improve the prognosis.

Since the human I-FABP gene has been reported previously (25), serum I-FABP could provide a valuable tool for the early and precise diagnosis of intestinal ischemia. The relationship between human intestinal diseases and serum I-FABP are subjects for future research.

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