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Release of heart fatty acid-binding protein into plasma after acute myocardial infarction in man

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Abstract

The release of cytoplasmic heart fatty acid-binding protein (H-FABP) into the plasma of cardiac patients up to 38 hr after the onset of the first clinical symptoms of acute myocardial infarction (AMI) was studied, using a sensitive direct and noncompetitive Enzyme Linked Immunosorbent Assay of the antigen capture type (sandwich ELISA), newly developed for the measurement of small amounts of human H-FABP in plasma samples. Plasma levels of H-FABP were compared with plasma activity levels of the myocardial cytoplasmic enzymes creatine kinase MB (CK-MB) and alpha-hydroxybutyrate dehydrogenase (α-HBDH). Upper normal levels of H-FABP (19 μg/l), CK-MB (10 U/l) and α-HBDH (160 U/l) as determined in plasma from 72 blood donors served as threshold levels. H-FABP levels were significantly elevated above their threshold level within 3 hr after AMI. Peak levels of H-FABP, CK-MB and α-HBDH were reached 4.1 ± 0.9 hr, 8.4 ± 1.4 hr and 25.0 ± 9.5 hr (means ± S.D., n = 10) after acute myocardial infarction, respectively. Serial time curves of the plasma contents of H-FABP reveal that after myocardial infarction H-FABP is released in substantial amounts from human hearts. In 18 out of 22 patients with established AMI the plasma FABP level was at or above the threshold level in blood-samples taken within 3.5 hr after the first onset of symptoms of AMI, while for CK-MB this applied to 9 patients and for α-HBDH to 6 patients. These findings suggest that for an early indication of acute myocardial infarction in man cytoplasmic heart fatty acid-binding protein is more suitable than heart type creatine kinase MB and/or alpha-hydroxybutyrate dehydrogenase. (Mol Cell Biochem **116:** 155–162, 1992)

Key words: fatty acid-binding protein, ELISA, acute myocardial infarction

Abbreviations: H-FABP – (cytoplasmic) Heart Fatty Acid-Binding Protein; LDH – Lactate Dehydrogenase, α-HBDH – α-Hydroxybutyrate Dehydrogenase; CK–MB – Creatine Kinase-MB; AMI – Acute Myocardial Infarction; PBS – Phosphate Buffered Saline; BSA – Bovine Serum Albumin

Introduction

Cytoplasmic fatty acid-binding protein (FABP) is one of the most abundant cytoplasmic proteins in the heart, as in human and rat heart it comprises about 15 and 30%, respectively, of the number of protein molecules present in the aqueous cytoplasm [1]. In a human heart this amounts to 0.5 mg of FABP per gram wet weight. Cytoplasmic FABP is thought to be involved in the uptake, transport and metabolism of fatty acids, but its

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precise role remains to be determined [for reviews see 2 and 3]. Previously heart-type FABP (H-FABP) has been shown to be released from isolated rat hearts upon cellular injury at a rate and to an extent similar to that of lactate dehydrogenase (LDH). [4]. The *in vivo* release of H-FABP from myocardial tissue upon cellular damage has subsequently been demonstrated immunochemically in rats, using polyclonal as well as monoclonal mouse antibodies against rat H-FABP [5], and in cardiac patients using monoclonal mouse antibodies against rabbit H-FABP [6]. Recently Tanaka *et al.* [7], using anti-human H-FABP antibodies in a competitive enzyme immunoassay, also showed elevated serum levels of FABP after acute myocardial infarction in man. These findings suggest that in cardiac patients the plasma FABP level can be used as an indicator for damage to myocardial tissue. The above observations warrant further investigations on the use of plasma FABP level for the (early) assessment of an acute myocardial infarction (AMI) in man.

In the present study we explored the concept [4] that the plasma level of FABP is an indicator especially for the early diagnosis of AMI. For a proper study of this concept we had to develop a sensitive and relatively disturbance-insensitive analytical method for the precise quantitation of H-FABP in human plasma. For this, and Enzyme Linked Immuno-Sorbent Assay (ELISA) of the antigen capture type (sandwich ELISA), in which antigen is bound by immobilized IgG and quantified with a second antibody-enzyme conjugate, appears the appropriate choice. Measurements of H-FABP and of the activities of creatine kinase (CK-MB) and α-hydroxybutyrate dehydrogenase (α-HBDH) were made in serial plasma-samples obtained from 26 patients with initial diagnosis of AMI. Levels of the specific proteins, determined in non-haemolytic plasma from 72 healthy individuals, served as threshold levels. The data indicate that for the early indication of AMI the plasma H-FABP level may be more suitable than that of CK-MB or α-HBDH.

Materials and methods

Patients

Twenty-six patients with an initial diagnosis of AMI were included in this study. AMI was positively diagnosed when patients showed elevation of more than one millimetre of the ST-segment in the recorded cardiogram and typical presentation of chest pain (often combined with radiation of pain to the left arm), in combination with transpiration, nausea, and/or shortness of breath. Diagnosis of AMI was confirmed by data on enzyme release (i.e. significant rise in LDH, CK and α-HBDH activity within 8 hr after onset of first symptoms) in 22 patients, the remaining 4 patients were diagnosed having unstable angina pectoris.

Blood sampling

Blood-samples were collected in glass tubes coated with ethylenediaminetetraacetic acid (EDTA) and subsequently spun for 10 min at 1500 × g. The resulting plasma was divided into several aliquots in glass tubes. Samples were stored at − 80° C until further use. Starting from admission of the patients to the coronary care unit of the hospital (Academic Hospital, AZM, Maastricht) blood-samples were taken based on a time schedule of 0, 4, 10, 18, 28 and 38 hr from admission (hospital routine). A subgroup of ten patients was sampled hourly during the first ten hours of hospitalization. Thereafter blood-samples were again taken in accordance with hospital routine up to 38 hr after admission, together resulting in 11–14 blood-samples per patient in this subgroup.

Non-haemolytic blood-samples of healthy donors (n = 72), collected at the local bloodbank, were used for the exploration of the threshold (= upper normal) levels in plasma of FABP, CK-MB and α-HBDH. In this study the threshold level is defined as the mean value plus twice the standard deviation of the particular protein, as measured in normal human plasma [8]. In addition, equal aliquots of each of the 72 normal human plasma samples were pooled. This pool plasma served as inter-assay control in the assays.

Isolation and purification of H-FABP

Human H-FABP, needed for the immunization of rabbits and for calibration of the immunoassay, was purified by gel permeation and anion-exchange chromatography as previously described [9]. Briefly, homogenates of human heart tissue (25%, w/v) were prepared in buffer, consisting of 10 mM Tris-HCl (pH 8.0), 150 mM KCl and 1 mM dithiothreitol. After centrifugation at 2,600 × g for 10 min and the supernatant at 105,000 × g for 90 min, the final supernatant (containing cytosolic proteins) was concentrated by ultra-filtration using a Diaflo YM5 membrane (Amicon, Danvers, MA, USA), and applied to a Sephacryl S200-SF column (Parmacia/LKB, Uppsala, Sweden) equilibrated with homogenization buffer. Elution of proteins was mon-

itored spectrophotometrically at 280 nm. FABP containing fractions, identified by Lipidex 1000 assay [10], were pooled, concentrated, dialyzed overnight at 4° C against 5 mM Tris-HCl (pH 8.0), and then applied to a Sepharose-Q fast flow column (Pharmacia/LKB, Uppsala, Sweden) equilibrated with dialysis-buffer, H-FABP was collected after stepwise gradient-elution (0–30 mM NaCl in 5 mM Tris-HCl, pH 8.0). Purity of H-FABP was assessed by SDS-PAGE and isoelectric focusing. Finally, the protein was dialysed against 10 mM K-phosphate (pH 7.4), containing 150 mM NaCl, and stored in aliquots at − 20° C.

Production of antibodies

Rabbits (Flemish giant) were immunized with 200 μg of pure human H-FABP each in a mixture of 2 ml PBS and Freund's Complete Adjuvant (FCA) (1:1, v/v), and were boosted in weeks 4 and 8 with antigen in a mixture of 2 ml PBS and Freund's Incomplete Adjuvant (FIA) (1:1, v/v). Two weeks after the last boosting blood was collected in glass tubes and spun for 10 min at 1500 × g. From the resulting polyclonal antiserum of IgG fraction was isolated by means of protein-A chromatography (Pharmacia/LKB, Uppsala, Sweden). Subsequently monospecific rabbit IgG against human H-FABP (αP-IgG) was isolated from this fraction by affinity chromatography, using a Sepharose column containing covalently bound human H-FABP. Monospecific rabbit IgG from one rabbit was conjugated with Horse Radish Peroxidase (αP-IgG-HRP) [11] to serve as the detector antibody in the sandwich ELISA.

Sandwich ELISA

In order to estimate H-FABP in plasma accurately we developed a sensitive direct noncompetitive Enzyme Linked Immunosorbent Assay of the antigen capture type (sandwich ELISA) essentially similar to that described by Börchers *et al.* [12] for the assay of H-FABP in subcellular fractions of bovine cardiac tissue. Our assay had to be more sensitive because, unlike subcellular tissue fractions, human plasma contains a relatively large amount of proteins (cf. albumin) in comparison to the amount of H-FABP. Thus, we developed an assay that would meet the requirements for measurement of minute amounts of human H-FABP in plasma samples. The assay was carried out in Falcon 3912 Micro Test III polystyrene microtiter plates (Becton Dickinson Labware, Oxnard, CA, USA). Each well was coated with 200 ng of αP-IgG (see above) in 50 μl 0.1 M ammonium carbonate (pH 8.8). Subsequently the wells

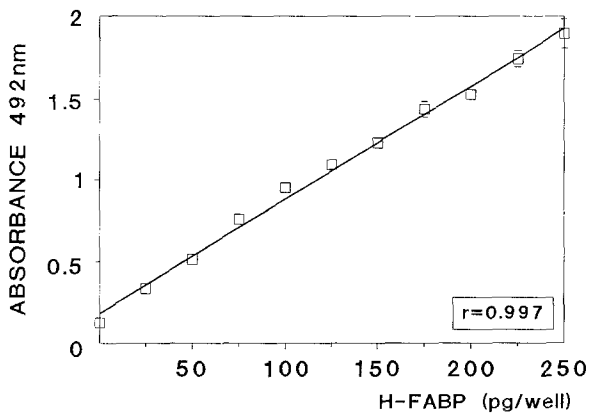

Fig. 1. Standard calibration curve for the sandwich enzyme linked immunosorbent assay (sandwich ELISA) for H-FABP. See text for details.

were washed with PBS containing 0.5% BSA and 0.05% Tween-20 (PBT) to remove all unbound IgG and at the same time saturate the wells, thereby preventing unspecific binding during the following steps of the assay. Next the standards and serially diluted (PBT) plasma samples were incubated in the wells for 1.5 hr at room temperature. After washing with PBT to remove unbound FABP the wells were incubated (1.5 hr at room temperature) with 70 ng of αP-IgG-HRP in 50 μl PBT for specific detection of bound FABP. Plates were developed with 100 μl substrate solution containing 10 mM ortho-phenylenediamine (OPD) and 0.008% H_2O_2, dissolved in 0.1 M sodium citrate (pH 5.0). Development was stopped with 50 μl 4N H_2SO_4 after 3–5 min and extinction was read at 492 nm using a MkII Plus microplate reader (Flow Lab, Bioggio, Switzerland).

Measurement of CK-MB and α-HBDH activity

Activity of CK-MB was measured by an enzyme immunoinhibition assay kit (# 418234, Boehringer, Mannheim, Germany). Activity of α-HBDH, which is predominantly LDH-isoenzyme-1, was measured spectrophotometrically with α-ketobutyrate as the substrate [13] using a commercially available test kit (# 161080, Boehringer, Mannheim, Germany).

Results

Sandwich ELISA

The calibration curve for the assay leveled beyond 500 pg H-FABP, but was found to be strictly linear

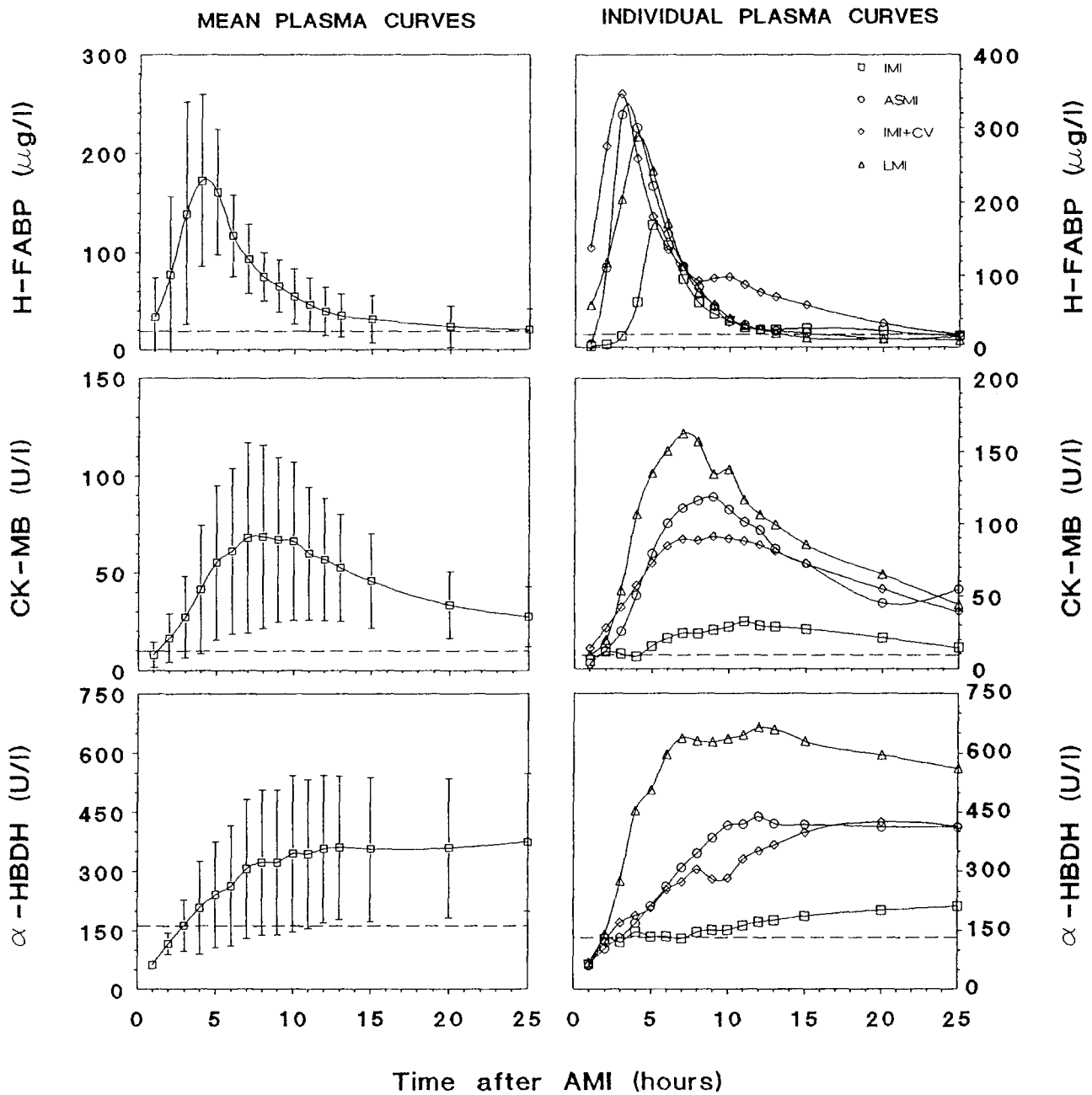

Fig. 2. Left-hand side panels: Mean plasma time-content curves of H-FABP and plasma time-activity curves of CK-MB and α-HBDH after acute myocardial infarction (n = 10). Right-hand side panels: Four typical examples of plasma curves. Each separate curve represents data from one patient. Curve IMI: Inferior myocardial infarction; Curve ASMI: Anteroseptal myocardial infarction; Curve IMI + CV: Inferior myocardial infarction in combination with Cardioversion; Curve LMI: Lateral myocardial infarction. Dotted lines in all panels indicate threshold levels of H-FABP, CK-MB and α-HBDH in normal human plasma (see text for details).

(R = 0.9970) within the range of 0 pg to 250 pg H-FABP (Fig. 1). All plasma samples studied were serially diluted with PBT, so as to assure that their H-FABP content would fall into the linear portion of the calibration curve. Recovery experiments (n = 11) using normal human plasma spiked with purified H-FABP yielded an average recovery of 93.5%. The interassay coefficient of variation was on the order of 6.5%.

Threshold levels

The plasma threshold levels for the three proteins studied, measured in plasma from 72 healthy volunteers,

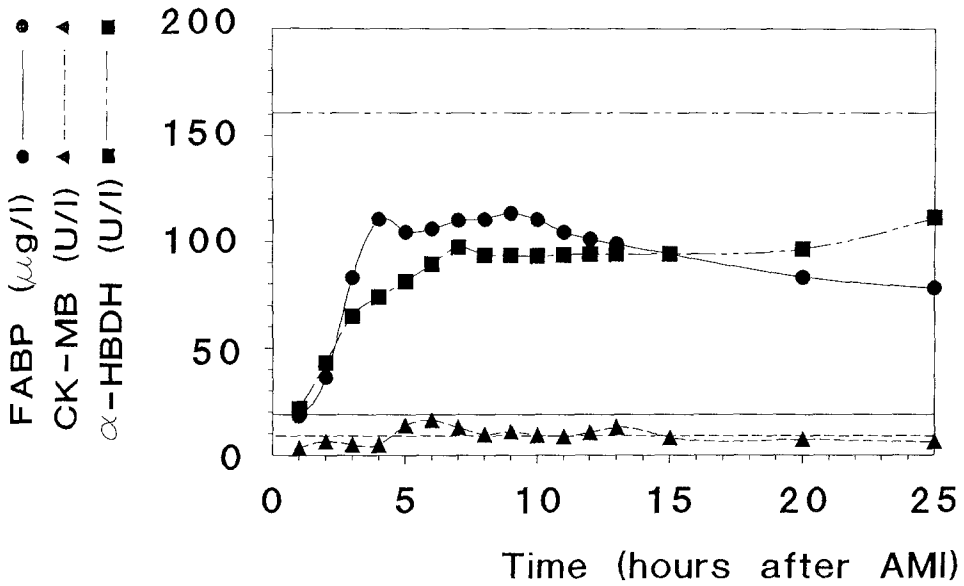

Fig. 3. Plasma levels of H-FABP, CK-MB and α-HBDH, and their respective thresholds (same line pattern) in a patient with inferior myocardial infarction in combination with instable angina pectoris and severe renal insufficiency, during 25 hr after the first onset of symptoms.

were calculated to amount to 19 μg/l for H-FABP, 10 U/l for CK-MB, and 160 U/l for α-HBDH.

Plasma curves

The time curves of the plasma content of FABP and the plasma activities of CK-MB and α-HBDH (Fig. 2) are the resultants of both the release into and the clearance from the circulation of these cytoplasmic proteins. In the left-hand side panels of Fig. 2 the mean release curves for FABP, CK-MB and α-HBDH of the subgroup of frequently sampled cardiac patients are presented. The plasma levels of FABP, CK-MB, and α-HBDH peaked at 4.1 ± 0.9 hr, 8.4 ± 1.4 hr and 25.0 ± 9.5 hr (means ± S.D., n = 10), respectively, after the onset of the first symptoms. In general relatively high plasma H-FABP levels in blood samples taken early are followed by relatively high levels of CK-MB and α-HBDH in the blood samples taken later, as is illustrated by typical release curves of individual patients (Fig. 2, right-hand side panels, which shows four typical release curves). The release curves for all 26 patients studied revealed that within 20 hr after the onset of the first symptoms, plasma H-FABP levels had returned to the threshold level (cf. Fig. 2). The disappearance rate constant of FABP from plasma (Kd) was calculated to range between 0.1 and 0.4 h^{-1}. In contrast, plasma activities of CK-MB and α-HBDH remained well above the threshold level until at least 25 hr after the onset of the first symptoms. Interestingly, one patient, who suffered from AMI in combination with unstable angina pectoris and severe renal insufficiency, showed a heightened (4 to 8 times the threshold level) FABP plasma level during the whole course of the sampling time (Fig. 3). The CK-MB plasma level in this case was only elevated 2 times the threshold level during a short period of the sampling time, while the α-HBDH plasma level stayed normal during sampling time.

Early diagnosis of AMI

In Fig. 4 the FABP plasma content and the CK-MB and α-HBDH plasma activities of the first blood-samples obtained (between 0.5 and 3.5 hr after AMI) from the 26 patients monitored in this study are plotted on a relative scale against their respective threshold levels. In 16 out of 22 patients with established AMI (for establishment of AMI see Material and methods), i.e. 73%, the plasma FABP level was already at or above the threshold level in these early blood-samples taken, while for CK-MB this was the case in only 9 (= 41%) and for α-HBDH in 6 (= 27%) patients (Fig. 4, closed symbols). Furthermore, of the six patients who showed no significant rise in plasma FABP level in the first blood-sample taken, two showed a significantly elevated level of FABP (not of CK-MB or α-HBDH) in a second blood-sample taken within 3.5 hr after AMI. The remaining four patients with established AMI showed significant rises in the plasmalevels of FABP, CK-MB, and α-HBDH in a next blood-sample taken

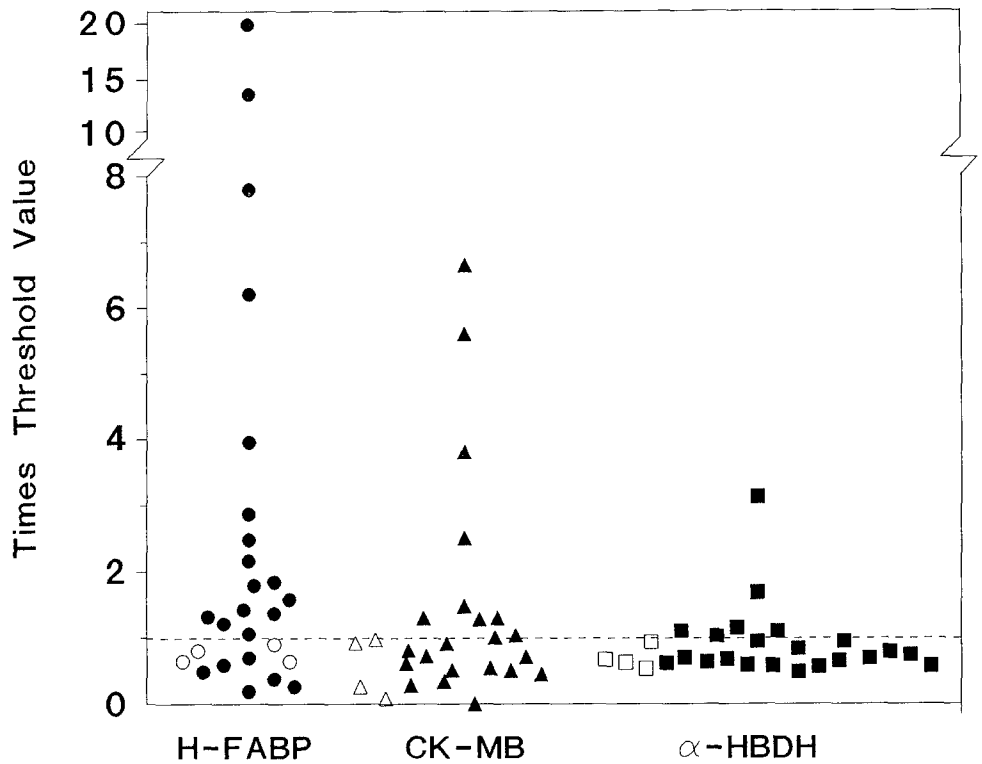

Fig. 4. Plasma levels of H-FABP, CK-MB and α-HBDH in *first* blood samples obtained (within 3.5 hr after onset of symptoms) from in total 26 cardiac patients in relative relation to their threshold level in plasma. Dotted line indicates threshold levels of H-FABP, CK-MB and α-HBDH in plasma. Solid symbols represent patients with established acute myocardial infarction (AMI, for establishment see Material and methods), open symbols refer to patients eventually diagnosed having instable angina pectoris.

between 3.75 and 7.75 hr after onset of the first symptoms. The four patients, from the total of 26, diagnosed as unstable Angina pectoris (Fig. 4, open symbols) showed no plasma levels of FABP, CK-MB or α-HBDH above their respective threshold level during sampling time. Thus, for the early (within 3.5 hr) diagnosis of AMI the plasma level of FABP appears to have been a useful extra tool in 18 out of the 22 established AMI patients (82%), that of CK-MB in 9 patients (41%) and that of α-HBDH in 6 patients (27%).

Discussion

In this study we demonstrate the accurate and reproducible measurement of H-FABP in serial plasma samples of cardiac patients with initial diagnosis of acute myocardial infarction (AMI), using a highly sensitive enzyme linked immunosorbent assay (ELISA) of the sandwich type, newly developed for the measurement of human H-FABP in plasma samples. Sensitivity and accuracy of the assay were evident from a linear calibration curve (between 0 and 250 pg H-FABP) and an almost complete recovery of purified H-FABP added to plasma samples. Comparison of the release of H-FABP into the circulation with that of CK-MB and α-HBDH revealed that the plasma level of the cytoplasmic heart type FABP can be used especially for the early diagnosis of AMI in man.

Plasma threshold levels

Measurement of H-FABP content in human plasma of 72 healthy individuals revealed a normal plasma level of $9 \pm 5\,\mu g/l$ (mean ± S.D.), leading to a threshold level of $19\,\mu g/l$ (mean + 2 S.D.). Assuming a total plasma volume of 3 litres, this means there is a constant pool of some $60\,\mu g$ of FABP, at the most, present in the bloodstream. Because FABPs from human heart and skeletal muscle are identical [14], and FABP from heart is not likely to be found in the bloodstream under normal conditions, the normal plasma level of FABP most probably stems for the greatest part from the contin-

uous release of FABP from damaged skeletal myocytes. Although the mean FABP content of skeletal muscle is relatively low, i.e. 0.2 mg per gram wet weight [14], the large total mass of striated muscle (about 40% of the body weight) makes that a human body contains about 6 grams of skeletal muscle FABP. Thus, the normal plasma level of FABP would represent less than 0.001% of this total amount.

The normal plasma FABP level found is of similar magnitude as the value (0–2.8 μg/l) reported by Tanaka *et al.* [7], but 3 to 4 orders of magnitude lower than the plasma levels of 15–60 mg/l given by Knowlton *et al.* [6]. However, the latter value seems very unlikely since it would imply that under normal circumstances the plasma pool of FABP equals that of 225–900 grams of skeletal muscle or 90–370 grams of cardiac muscle.

FABP as indicator for AMI

Comparison of the time curves of the plasma content of FABP and of the plasma activities of CK-MB and α-HBDH reveals that FABP is released into and cleared from the circulation more rapidly than is CK-MB and α-HBDH. The rapid clearing will relate to the fact that small molecules like H-FABP (15 kDa) are easily cleared from the circulation by urinary excretion [15–17] unlike larger molecules such as CK-MB (80 kDa) or α-HBDH (138 kDa) which stay in the circulation for a longer period of time. With our immunoassay we were indeed able to qualitatively establish the presence of FABP in urine collected from some patients during their hospitalization, thus confirming urinary excretion of FABP, similar to findings of Tanaka *et al.* [7]. In addition, the patient with renal insufficiency showed elevated plasma FABP levels for a prolonged period of time. Nevertheless, our preliminary and qualitative data on renal excretion of FABP and also bearing the probable inter-patient variation in mind, do not justify any conclusions about clearance rates of FABP from the bloodstream as yet. The relatively short half-life time of FABP in plasma is helpful in maintaining a low normal c.q. low threshold plasma level. In turn, as demonstrated in this study, the relatively low threshold plasma level will permit the discrimination of elevated plasma levels at an earlier point in time after AMI than the discrimination of elevated CK-MB or α-HBDH levels from their respective threshold values. The rapid release of FABP into the circulation seems to be independent of the site of myocardial damage in the heart (see Fig. 2, right-hand side panels).

Furthermore, its low threshold level and rapid clearing from plasma makes the plasma level of FABP not only useful for the early assessment of AMI, but presumably also for the demonstration of the occurrence of a possible recurrent infarction.

Myoglobin, the oxygen-carrying protein inside muscle cells, has also been introduced as a plasma marker for early indexation and quantification of acute myocardial infarction [18–25]. However, the discrimination between skeletal muscle and cardiac tissue damage will be more difficult with myoglobin than with H-FABP, since the skeletal muscle myoglobin content is approximately twice that of the heart [26, 27] while the FABP concentration is striated muscle is only 10–50% of that in human cardiac muscle [28]. Hence, interference on an assay from skeletal muscle damage will be 4-20 times less severe in case of an FABP-measuring assay, compared to a myoglobin-measuring assay. On the other hand, for both indicators significant interference will take place only if AMI and extensive skeletal muscle injury occur simultaneously within a few hours.

Concluding remarks

The plasma level of H-FABP was shown to be a useful parameter for the assessment or exclusion of acute myocardial infarction in man, in addition to the commonly used parameters. Furthermore, as a plasma marker for damage to myocardial tissue H-FABP was found to have advantages over those of other protein markers (i.e. CK-MB and α-HBDH) as it appears to be suitable especially for the early (within a few hours) detection of AMI and presumably also for the monitoring of a possible second infarction. The reported sandwich ELISA, when adapted for more rapid clinical application, could become a valuable tool for the early assessment of AMI. In addition to its use for clinical diagnostic purposes, the use of the plasma FABP level as early marker of myocardial infarction will also be of interest for various types of experimental studies in which cardiac tissue damage (may) occur(s).

In view of the existence of other tissue specific types of cytoplasmic FABP, i.e. in kidney, liver, intestine, brain, and adipose tissue [1, 3], cellular injury in these tissues may be specifically and accurately assessed by a similar approach.

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