

Identification of serum proteins that bind with S100A8, S100A9 and S100A8/A9: clinical significance of using proteins for monitoring the postoperative condition of liver recipients

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Abstract

Background: Serum proteins that non-specifically bind with human S100A8/A9 (h-S100A8/A9) have been proposed. Our aim was to isolate and identify these proteins, and verify their clinical significance for monitoring the postoperative condition of liver recipients, and further to discuss the transportation of human fibronectin (h-FN) with h-S100A8/A9 and its functional role *in vivo*.

Methods: To isolate the serum proteins, recombinant human S100A8, S100A9 and S100A8/A9 affinity columns were used. Proteins were identified by mass spectrometry. Two enzyme-linked immunosorbent assays (ELISA) were used to measure h-S100A8/A9 and h-FN in the sera of liver recipients. Flow cytometry was employed to detect h-S100A8/A9 and h-FN on immunological cells. Western blotting was used to confirm serum constituents using antibodies specific to each constituent.

Results: One of the proteins was identified with h-FN, and its fluctuation pattern in the serum of the recipient was in contrast to that of CRP. Flow cytometry showed a positive reaction for h-S100A8/A9 and h-FN on neutrophils and monocytes, indicating that both proteins exist on these cells.

Conclusions: The h-FN could be transported with S100A8/A9 in blood and/or on immunological cells, and effectively prevent further attack by various internal oxidants or repair damaged liver tissue *in vivo*.

Keywords: S100 proteins; fibronectin; inflammation; neutrophil; macrophage, CRP

1. Introduction

Human S100 proteins, h-S100A8, h-S100A9 and h-S100A8/A9, were first found in the synovial fluid of patients with rheumatoid arthritis [1]. S100 proteins are characterized by their origin and immunological properties in activated neutrophils and/or macrophages

[2-6]. To date, a variety of their immunological functions in inflammation have been subjected to investigation by many researchers. Although S100 proteins are reportedly involved in the induction of acute inflammation, their immunological properties have not necessarily been clarified, which is of note [7-11]. We recently reported that among S100 proteins, h-S100A8/A9 was markedly increased in the serum of a recipient of small intestine transplantation with acute inflammation associated with rejection [12]. In this case, fluctuation of the serum level of S100A8/A9 did not correspond to that of C-reactive protein (CRP), indicating that the functional role of h-S100A8/A9 is different from that of CRP. h-S100A8/A9 binds with heparin, heparin sulfate glycosaminoglycans on endothelial cells, and arachidonic acid with high affinity, indicating a potential property that is essentially different from that of CRP [13,14]. Against such a background, we hypothesized the existence of other constituents binding with h-S100A8/A9 in the human serum. In this study, we describe another potential property of h-S100A8/9 and its interaction with serum constituents, and present data supporting its functional role.

2. Materials and methods

2.1. Materials

SephacrylTM S-300 HR was purchased from Pharmacia Co. Ltd., Uppsala, Sweden; human serum fibronectin (h-FN) was obtained from BD Biosciences Co. Ltd., San Jose, CA; fluorescein 5-isothiocyanate (FITC) and lipopolysacchride (LPS) were from Sigma Chemical, Co. Ltd., St. Louis, MO; streptavidin (STA)-horseradish peroxidase (HRP), STA-Texas Red and anti-rabbit IgG (goat) IgG-HRP conjugates, and biotin (Long arm)-NHS were obtained from Vector Co. Ltd., Burlingame, CA; all others were from Nacalai Tesque or Wakenyaku Co. Ltd., Kyoto, Japan.

2.2. Methods

2.2.1. Expression of cDNA for h-S100A8 or h-S100A9

cDNAs with histidine tag sequences for h-S100A8 and h-S100A9 were synthesized using common gene technology, including polymerase chain reaction (PCR). The two cDNAs were each inserted into a pCold-I vector (Takara Bio Co. Ltd., Kyoto, Japan) [15]. The two vectors were transformed into *E. coli* cells (BL21), respectively. The recombinant cells were cultivated in a large culture bottle (approx. 8 liters of LB broth) at 37 °C for 12 h. When the absorbance of the culture medium at 600 nm reached 0.5 to 0.8, the temperature in the bottle was quickly reduced to 15 °C. *E. coli* cells were further cultivated for 24 h at the same temperature after adding 1 mmol/l isopropyl- β -D(-)-thiogalactopyranoside as the final concentration. The cells were harvested and washed once with 50 mmol/l Tri-HCl buffer (pH7.4)/0.15 mol/l NaCl, and kept frozen at -80 °C until use.

2.2.2. Purification of recombinant human S100A8 and S100A9

E. coli cells (approx. 5 g) expressing recombinant human S100A8 (r-S100A8) or S100A9 (r-S100A9) were re-suspended in 0.25 liters of 10 mmol/l imidazole/0.3 mol/l NaCl/50 mmol/l phosphate buffer (pH8.0) for 1 h at room temperature, and then treated using an ultrasonic generator for 10 to 15 min at 4 °C. After centrifugation at 18560 \times g for 20 min at 4 °C, clear supernatant was applied to an Ni-agarose affinity column (19 \times 50 mm) equilibrated with the same buffer solution. After washing with 20 mmol/l imidazole/0.3 mol/l NaCl/50 mmol/l phosphate buffer (pH8.0) thoroughly, r-S100A8 or r-S100A9 was eluted from each column with 0.25 mol/l imidazole/0.3 mol/l NaCl/50 mmol/l phosphate buffer (pH8.0). Major fractions containing r-S100A8 or r-S100A9 were collected and then dialyzed against 50 mmol/l phosphate buffer (pH8.0)/0.3 mol/l NaCl at 4 °C overnight, and kept frozen at -80 °C until use. The concentrations of the products were determined by the method of Lowry *et al.* using bovine serum albumin as a standard [16].

2.2.3. Preparation and purification of recombinant human S100A8/A9

r-S100A8 was mixed with r-S100A9 at a mole ratio of approximately one. The mixture was placed in a dialysis tube followed by incubation in dialyzed against 2.0 mol/l Tris-NaOH solution (pH12) at 4 °C overnight. Recombinant human S100A8/A9 (r-S100A8/A9) was purified through a gel filtration column embedded with SephacrylTM S-300 HR (25 \times 1000 mm) and equilibrated with 50 mmol/l Tris-HCl buffer (pH10)/0.3 mol/l NaCl. Major fractions containing r-S100A8/A9 were collected and concentrated to an adequate volume, and kept frozen at -80 °C until use.

2.2.4. SDS-PAGE and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the absence of 2-mercaptoethanol (2-ME) according to the

method of Towbin *et al.* [17]. Proteins in the gel were electrophoretically transferred to a sheet of nitrocellulose membrane. Western blotting was also carried out to confirm the cross-reactivity of anti-h-S100A8/A9 antibody with r-S100A8/A9. An antibody-bound protein band was finally visualized as described previously [12].

2.2.5. Isolation of serum proteins that bind with r-S100 proteins from the sera of patients

Human pooled serum from many patients was preliminarily diluted about three-fold with 50 mmol/l phosphate buffer (pH7.2)/0.15 mol/l NaCl (buffer A). One hundred milliliters of the working pooled serum was applied to r-S100A8-, r-S100A9- and r-S100A8/A9-Sepharose 4B columns (19 \times 50 mm), respectively. After washing with buffer A, proteins were eluted from each column with 0.2 mol/l glycine buffer (pH2.3) and concentrated to an adequate volume. The proteins were dialyzed against 2 liters of buffer A at 4 °C overnight and then kept frozen at -80 °C until use.

2.2.6. Preparation of antibodies directed to serum proteins binding with r-S100 proteins

Antisera were raised in rabbits against serum proteins that bind with r-S100 proteins [12]. From the antiserum, partially purified IgG was obtained by precipitation with 60% saturated ammonium sulfate and DEAE-cellulose (DE52) chromatography. Fractions containing IgG were collected and mixed with 7.7 mmol/l sodium azide as the final concentration. Anti-h-FN IgG was affinity-purified using an h-FN-Sepharose 4B column [12].

2.2.7. Mass spectrometry

Mass spectrometric identification of proteins was performed as previously described [18]. Briefly, after SDS-PAGE, proteins were visualized by CBB staining and excised separately from gels, followed by in-gel digestion with trypsin (Promega Corporation) in 50 mmol/l ammonium bicarbonate buffer (pH8.0) containing 2 % acetonitrile overnight at 37 °C. Molecular mass analyses of tryptic peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using a ultraflex TOF/TOF (Bruker Daltonics). Proteins were identified by comparing the molecular weights determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in NCBI nr.

2.2.8. Biotin and FITC labeling

Biotinylation of proteins with biotin (Long-arm)-NHS and FITC labeling was carried out according to the manufacturer's instructions. Unbound biotin-NHS was dialyzed for 12 h at 4 °C against 2 liters of buffer A. The procedures were repeated twice, replacing the 2 liters of buffer A. The labeled products were kept in a refrigerator until use. Unbound FITC was removed through a Sephadex G-25 column (15 \times 600 mm) for gel filtration in the dark. Major fractions

containing FITC-labeled proteins were collected, concentrated and then kept in a refrigerator in the dark.

2.2.9. Measurement of h-FN, h-S100A8/A9 and another laboratory markers

To measure h-FN in the sera of liver recipients, we newly developed an enzyme-linked immunosorbent assay (ELISA) for h-FN, as described previously [12]. Except for immobilization of the primary antibody, all procedures were carried out at room temperature. Briefly, the procedures were as follows: 25 μ l of serum sample (500-fold dilution with Blocking One; Nacalai Tesque Co. Ltd.) was added to each well of a 96-well polycarbonate plate, which was preliminarily coated with anti-h-FN (rabbit) IgG (5 mg/l in 75 mmol/l carbonate buffer, pH9.5), followed by incubation for 1 h to allow immunological reaction. After washing, 100 μ l of anti-h-FN (rabbit) IgG-biotin conjugate, diluted adequately with the standard matrix solution, was added to each well of the plate, followed by further incubation for 1 h. The plate was washed again as above with the same washing solution, and 100 μ l of STA-HRP conjugate was added to all wells of the plate, and further incubated for 30 min to form the biotin-STA-HRP complex. Finally, HRP activity was determined colorimetrically. h-S100A8/A9 was measured by ELISA. For comparison, other serum markers, such as CRP, AST and ALT, were also determined using an automatic chemical analyzer.

2.2.10. Flow cytometry

Sample preparation was carried out as follows: human leukocytes from many healthy volunteers were separated, as described previously [12], and re-suspended at about 5×10^6 /ml in Hank's Balanced Salt Solution (HBSS). All cells were blocked with rabbit IgG from non-immunized rabbits (10 μ g/ml as a final concentration) for 20 min on ice. Some of the cells was used as a negative control, and residual cells were stained with anti-h-S100A8/A9 IgG-, anti-h-FN IgG-, and h-FN-FITC conjugates, respectively, for 30 min on ice. h-FN-FITC conjugate was used to examine whether h-FN binds to immunological cells, such as neutrophils and monocytes. All cells were finally re-suspended in an adequate volume of the same solution, and then analyzed by flow cytometry using FACSvantage (BD Biosciences Co. Ltd., Tokyo, Japan) equipped with CellQuest software.

2.2.11. Possible existence of h-FN-h-S100A8/A9 complex

The existence of the h-FN-h-S100A8/A9 complex in the sera of liver recipients was examined using an anti-h-FN (rabbit) IgG-Sepharose 4B column, based on its immunological properties. Briefly, pooled serum of the recipients, which was adequately diluted with buffer A, was applied to the column. After washing with buffer A, proteins were eluted with 0.2 mol/l glycine buffer (pH2.3), and the pH of the solution was quickly adjusted to 7.0 with 2.0 mol/l NaOH. To detect the h-FN-h-S100A8/A9 complex, ELISA for h-S100A8/A9,

in which anti-h-S100A8/A9 monoclonal antibody (Mo2B9) was used as the first antibody, was employed [12]. Anti-h-FN IgG-biotin complex with STA-HRP was used to detect h-FN-h-S100A8/A9 complex as a second antibody. In addition, anti-h-S100A8/A9 Fab'-HRP conjugate was also used to determine free h-S100A8/A9 as a second antibody.

2.2.12. Statistical analysis

Statistical analysis was performed using the parametric test for pair-wise comparisons with controls. Significant differences between groups were identified using Student's *t*-test (for the difference between two means). Values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Preparation of r-S100A8, r-S100A9 and r-S100A8/A9

Both r-S100A8 and r-S100A9 were successfully expressed in *E. coli* cells. As shown in Figure 1, the purity of r-S100A8 and r-S100A9 together was $\sim 95\%$, as determined visually (Fig. 1A, lanes a and b). Preparation of r-S100A8/A9 was also successfully achieved *in vitro*. Crude r-S100A8/A9 was purified using a gel filtration column (see *Methods*). When examined by SDS-PAGE, the purity was $>95\%$ visually (Fig. 1B, lane d).

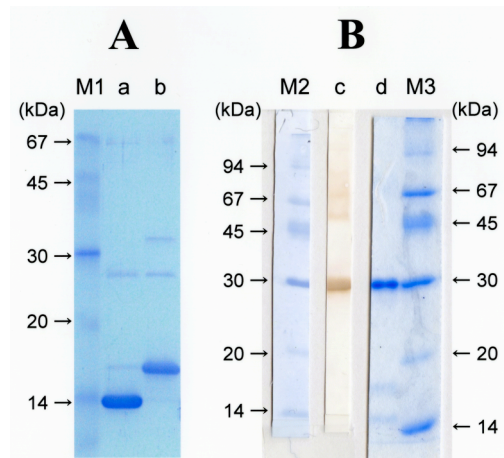


Fig. 1. Preparation and purification of r-S100A8, r-S100A9 and r-S100A8/A9

SDS-PAGE was carried out in the presence (Fig. 1A) and absence (Fig. 1B) of 2-ME. In A, lane M1 shows molecular mass markers. Lanes a and b show purified r-S100A8 and r-S100A9, respectively. In B, lanes M2 and M3 show molecular mass markers. Lanes c and d contain purified r-S100A8/A9 (main band), and r-S100A8 and S100A9 monomers (minor bands). Anti-h-S100A8/A9 (rabbit) IgG was used as the first antibody and anti-rabbit IgG (goat) IgG-HRP conjugate as the second antibody (lane c).

3.2. Reactivity of anti-h-S100A8/A9 antibody with r-S100A8/A9

Anti-h-S100A8/A9 antibody apparently reacted with r-S100A8/A9, but not with r-S100A8 or r-S100A9 monomer (Fig. 1B, lane c), indicating that the antibody could recognize the three-dimensional structure of

r-S100A8/A9. As a result, r-S100A8/A9 was practically equivalent to h-S100A8/A9 [12].

3.3. Identification by mass spectrometry

Serum proteins that bound with r-S100A8, r-S100A9 and r-S100A8/A9 were isolated from the sera of many patients using each affinity column (see *Methods*). As examined by SDS-PAGE in the presence of 2-ME, various proteins were contained in the sample from each column (Fig. 2A, lanes a-c). Interestingly, these proteins were eluted with 0.2 mol/l glycine buffer (pH2.3), but not with 9 mmol/l acetic acid, indicating that they bound tightly, to some extent, to r-S100A8, r-S100A9, and r-S100A8/A9 affinity columns. As confirmed by Western blotting, antibodies directed to these serum proteins positively reacted with many protein bands (Fig. 2B, lanes a-c). Among them, fourteen proteins, including their subunits, corresponding to the positive bands were identified by mass spectrometry (Fig. 3, p1~p14), one of which was serum fibronectin (Table 1). Also of note, some coagulation-related factors were identified.

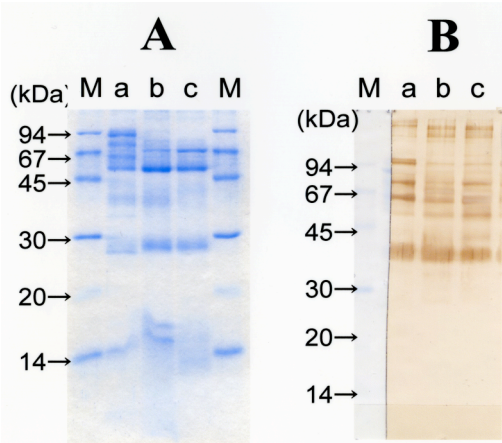


Fig. 2. Isolation of serum constituents binding with r-S100A8/A9 from human sera of patients

In A, SDS-PAGE for serum constituents binding with r-S100A8/A9 was carried out in the presence of 2-ME. Lane M shows molecular mass markers. Lanes 1, 2 and 3 show protein bands of the serum constituents eluted from r-S100A8-, r-S100A9- and r-S100A8/A9-Sepharose 4B affinity columns, respectively. In B, anti-serum constituents antibodies preliminary raised in rabbits and anti-rabbit IgG (goat) IgG-HRP were used as the first and second antibodies, respectively. Loaded samples of lanes a, b and c in panel A correspond to those in panel B, respectively.

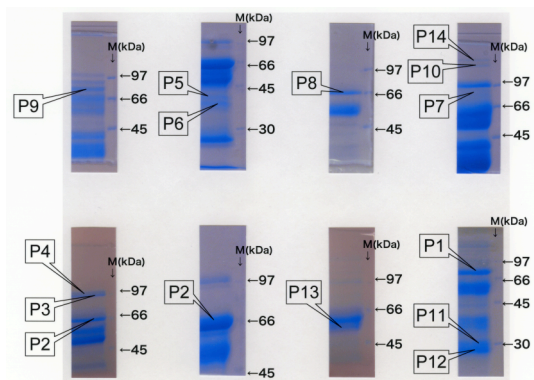


Fig. 3. Protein bands of serum constituents identified by mass spectrometry

SDS-PAGE was carried out using polyacrylamide gels of 100 g/l, 125 g/l or 150 g/l in the presence of 2-ME. Proteins were stained with Coomassie brilliant blue R-250. Protein bands of the serum constituents (P1 to P14) were identified by mass spectrometry.

Table 1

Identification of PXs by mass spectrometry

	Proteins identified
P1	IgM chain C region
P2	Serum albumin
P3	Plasminogen
P4	Plasminogen
P5	Plasminogen and Fibrinogen beta chain
P6	Plasminogen
P7	Plasminogen and Fibulin-1
P8	Serum albumin
P9	IgM chain C region and Antithrombin-III
P10	Fibronectin
P11	Immunoglobulin light chain
P12	Immunoglobulin light chain
P13	Antithrombin-III/IGHA1 ^a and Inter-alpha-trypsin inhibitor heavy chain H4
P14	Fibronectin

a: Ig alpha-1 chain C region

3.4. Interaction between h-FN and h-S100A8/A9 in vitro

It was examined whether h-S100A8/A9 binds to an h-FN-Sepharose 4B column. As determined by ELISA for h-S100A8/A9, the reaction was apparently positive, indicating that h-S100A8/A9 exactly binds to the affinity column (Figs. 4A and 4B, lane E). This strongly suggests that h-S100A8/A9 interacts with h-FN to form a complex *in vivo*.

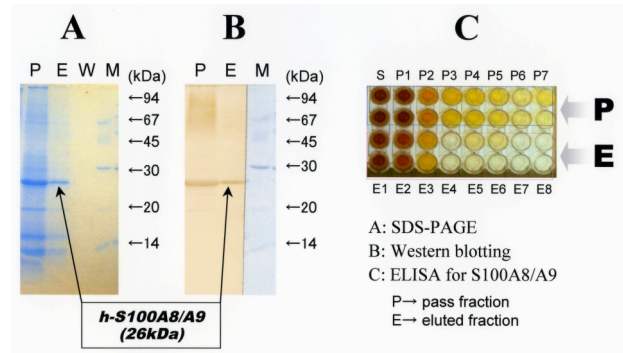


Fig. 4. Interaction between h-FN and h-S100A8/A9 in vitro

Examination of interaction between h-FN and h-S100A8/A9 in vitro was carried out using the h-FN-Sepharose 4B affinity column. 1.5 ml of h-S100A8/A9 (approx. 1 mg) was applied to the column. h-S100A8/A9 bound to the column was eluted with 0.2 mol/l glycine buffer (pH2.3). Panels A, B and C show SDS-PAGE, Western blotting and ELISA, respectively. P and E in all panels indicate pass and elution fractions from the column, respectively. Lane M shows molecular mass markers. Panel C shows ELISA for h-S100A8/A9, in which S indicated h-S100A8/A9 before its application to the column. P1~P7 indicate pass fractions from the column. W indicates a pass fraction of washing solution. E1~E7 are eluates from the column.

3.5. Possible h-FN-h-S100A8/A9 complex formation in serum

To measure free h-S100A8/A9 in the eluate, ELISA for h-S100A8/A9 was carried out (see *Methods*). In ELISA, the reaction was apparently positive and linear for h-S100A8/A9 in proportion to the dilution ratio of the eluate (Fig. 5A). Meanwhile, when anti-h-FN antibody-biotin-STA-HRP complex was used as the second antibody in ELISA, it also showed a positive reaction (Fig. 5B). These data indicate that free S100A8/A9 and h-FN-h-S100A8/A9 complex exist together in the eluate due to the immunological properties of the antibodies used in this study.

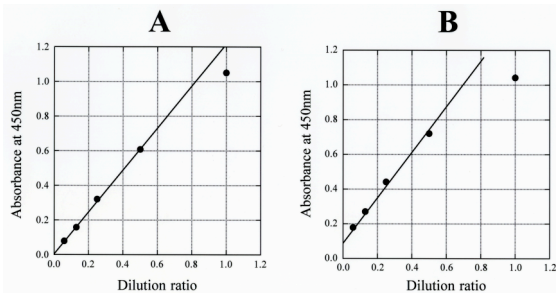


Fig. 5. Examination of h-FN-h-S100A8/A9 complexes by ELISA. To isolate h-FN-h-S100A8/A9 complex in the sera of liver recipients, an anti-h-FN IgG-Sepharose 4B column was used. In A, free h-S100A8/A9 was determined by the ELISA previously described [13]. In B, anti-h-FN IgG-biotin conjugate, together with STA-HRP, was used as the second antibody to capture the h-FN component of the complex in the eluate. In A and B, a two-fold dilution series of the eluate was used to detect the complex.

3.6. Linearity and sensitivity of ELISA for h-FN, and its precision

We newly developed an ELISA for h-FN as described in *Methods*. A novel standard curve was obtained using 2 mg/l h-FN standard (BD Biosciences Co. Ltd.) (Fig. 6A). Under the standard conditions, the ELISA reaction was linear for h-FN from 0 to 930 $\mu\text{g/l}$ (Fig. 6B). We assessed the reproducibility of the method using purified h-FN and pooled sera from healthy individuals or patients with various liver diseases. Within-day CVs were in the range of 3.0% to 5.2%, and between-day CVs were between 5.5% and 11.2%, indicating the fairly good reproducibility of this ELISA. The recovery of endogenous plus exogenous h-FN was 108% (mean), with a range of 96% to 120%. The normal reference interval, as estimated by this method with sera obtained from 103 healthy individuals [48 men and 56 women; age 18-35 (mean 24) years], was $366 \text{ mg/l} \pm 19.1 \text{ mg/l}$ (mean \pm 2SD).

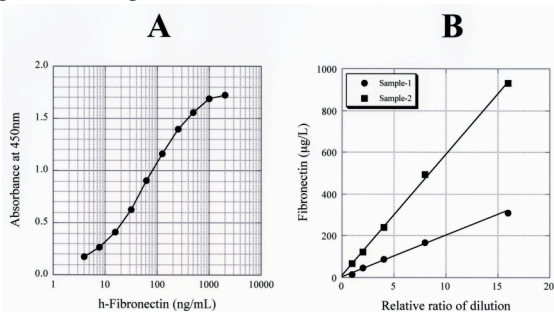


Fig. 6. Linearity and sensitivity of the ELISA for h-FN, and its precision and recovery

In A, h-FN (BD Biosciences Co. Ltd.) was used as the standard. In B, two pooled sera with different concentrations (low and high) were used to confirm the linearity of the ELISA.

3.7. Application of ELISA for h-FN to clinical samples

To assess the clinical value of our new ELISA for h-FN, we examined sera obtained from five liver recipients. Among them, data from a representative recipient are presented in this study. For comparison, the presence of other markers, such as h-S100A8/A9, CRP, AST and ALT, in serum were also determined.

The patient (a 63-year-old man), hospitalized with hepatocellular carcinoma, underwent liver transplantation from a living-related donor. Prominent peaks for AST and ALT were seen immediately after the operation, accompanied with h-S100A8/A9 and CRP, but subsequently disappeared within one week (Fig. 7). The level of CRP began to markedly increase a little later, transiently maintained a high level in the serum, and then gradually decreased (*horizontal bar*). Meanwhile, an increase in the h-S100A8/A9 level was seen little after that of CRP and soon peaked, gradually decreasing a month later. The level of h-FN, however, began to decrease immediately after the operation and remained low until it increased about two weeks after surgery. Thus, the fluctuation pattern of the serum level of h-FN was almost in contrast to that of CRP.

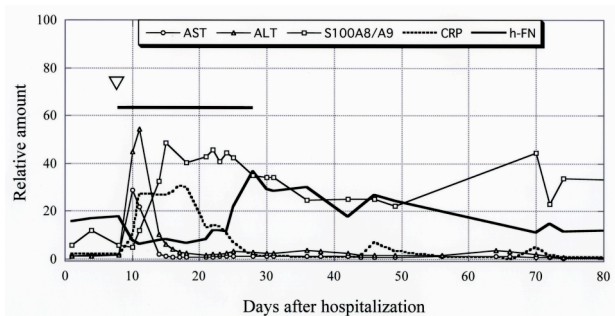


Fig. 7. Changes in h-FN, h-S100A8/A9, CRP and other laboratory markers in serum of a liver recipient

Liver transplantation was performed for hepatocellular carcinoma associated with liver cirrhosis (c-type). Concentrations of h-S100A8/A9 and h-FN were measured by the ELISA methods for each protein, respectively. Other laboratory markers were measured using an automatic chemical analyzer (TBA200FR-Neo; TOSHIBA, Tokyo). The day of surgery is indicated by a vertical open triangle. Values are presented as the relative ratio to the upper value of the normal range of each marker.

3.8. Observation of h-S100A8/A9 and h-FN on immunological cells

As examined by flow cytometry, the h-S100A8/A9 reaction was apparently positive on neutrophils and monocytes, whereas that for h-FN was lower, indicating however that both h-S100A8/A9 and h-FN exist on these immunological cells (Fig. 8, panels N1 and M1). On the other hand, no positive reaction for either protein was seen on lymphocytes (Fig. 8, panel L1). In addition, h-FN bound to both neutrophils and monocytes, but not

to lymphocytes (Fig. 8, panels N2, M2 and L2).

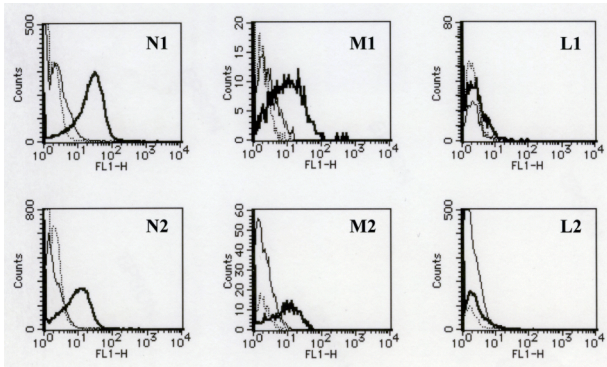


Fig. 8. Detection of h-FN and h-S100A8/A9 on surface membrane of immunological cells

Panels N1 and N2 show neutrophils. Panels M1 and M2 show monocytes. Panels L1 and L2 show lymphocytes. In all panels, dotted line indicates negative control. In panels N1, M1 and L1, heavy and thin solid lines indicate h-S100A8/A9 and h-FN, respectively. In panels N2, M2 and L2, heavy and thin solid lines indicate h-FN and human albumin (internal control).

4. Discussion

In this paper, we have described the isolation and identification of new serum constituents that non-specifically bind with r-S100A8-, r-S100A9- and r-S100A8/A9-Sepharose 4B columns. In addition, we have mentioned the potential of h-S100A8/A9 as a carrier protein for serum constituents and presented data supporting its functional role. h-S100A8/A9 is closely involved in acute inflammation because the production and secretion of h-S100A8/A9 is almost entirely limited to activated neutrophils and macrophages in acute inflammation [19]. Thus, the value of h-S100A8/A9 *in vivo* is based on its origin and immunological properties.

The property of h-FN to bind tightly, under some conditions, with an r-S100A8/A9 affinity column *in vitro* may be helpful for understanding its functional role *in vivo*. The interaction of h-FN with h-S100A8/A9 is noteworthy because h-FN is reportedly a protein that binds with various constituents *in vivo*. In the present study, we demonstrated the existence of h-S100A8/A9 and h-FN on immunological cells, such as neutrophils and monocytes, or the adhesion of h-FN to both cells, as well as the interaction of h-S100A8/A9 and h-FN with high affinity *in vitro* (Figs. 4, 5 and 8). These data strongly support our hypothesis that h-FN could be transported with h-S100A8/A9 in blood and/or on immunological cells. Transported h-FN is consumed after partial hepatectomy to prevent further attack by various internal oxidants or to repair damaged tissues during and after surgery, although its mechanism remains unclear [20]. For elucidation, we focused on the postoperative fluctuation of h-FN levels in the sera of liver recipients, together with other laboratory markers. Interestingly, the fluctuation pattern was in contrast to that of the CRP level for a short period (Fig. 6, horizontal bar). As described above, this probably reflects the possibility of h-FN being consumed to protect the liver graft after transplantation. Thus, we

found that h-FN is clinically another useful biomarker for monitoring the postoperative condition of liver recipients, and that h-S100A8/A9 could serve as its carrier protein *in vivo* in the acute inflammatory phase.

Comprehensive elucidation of the immunological properties of h-S100A8/A9 has long been desired, but it has not necessarily been achieved because of the complication of immune reactions *in vivo*; however it was reported that S-nitrosylated S100A8 is a protein with anti-inflammatory properties [21]. This supports our hypothesis concerning the functional role of h-S100A8/A9 in acute inflammation. Recently, microscopic images showed the co-localization of h-S100A8/A9 and h-FN on immunological cells, and the accumulation of h-FN, which may be diffusely released from the cells, bound with damaged liver tissue (data not shown). Integrin commonly exists on endothelial cells of capillary vessels and adheres to various serum constituents, functioning as a mediator on the cells [22,23]. h-FN transported to endothelial cells with h-S100A8/A9 and/or on immunological cells may be further translocated near to an inflamed area via such an integrin or these cells, although the mechanism remains unclear. In the present study, we also mentioned a mechanism for understanding the effect of h-FN on suppression of the inflammatory reaction in damaged liver tissue.

On the other hand, it is noteworthy that several coagulation factors, such as antithrombin III, plasminogen and fibrinogen, were identified in this study (Table 1). Thus, h-S100A8/A9 seems to be closely related to pathological conditions, such as the disseminated intravascular coagulation phenomenon (DIC) that probably occurs, to a greater or lesser extent, in the bloodstream after liver transplantation. Interaction between h-S100A8/A9 and these coagulant factors *in vivo* may show an influence on the DIC, which is of note. Indeed, marked fluctuation of the level of antithrombin III, measured by ELISA, in the serum of a liver recipient was observed (data not shown). If the DIC is in an advanced stage, the condition of a liver recipient may deteriorate postoperatively. It is therefore clinically very important to reduce DIC as early as possible. Recently, h-S100A8/A9-serum constituent complexes, isolated from the serum of patients using an anti-h-S100A8/A9 IgG-Sepharose 4B column, were clearly demonstrated by SDS-PAGE in the presence and absence of 2-ME (data not shown). When antibodies directed to serum constituents that bind with r-S100 proteins were used, Western blotting showed a positive reaction, indicating that coagulation factors were contained in these complexes. This may enable the preparation of monoclonal antibodies against epitopes of the three-dimensional structure of h-S100A8/A9-coagulation factor complexes, and to develop an ELISA for these complexes. Our current investigations are focusing on the preparation of such monoclonal antibodies specific to these complexes.

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Abbreviations

h-FN, human fibronectin; LPS, lipopolysaccharide; FITC, fluorescein 5-isothiocyanate; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; 2-ME, 2-mercaptoethanol; STA, streptavidin; HRP, horseradish peroxidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; DIC, disseminated intravascular coagulation.

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