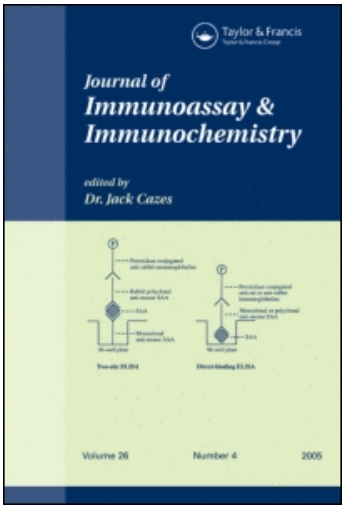

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Human Serum Contains Detectable Levels of the Hsp70 Cochaperone HspBP1 and Antibodies Bound to HspBP1

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Abstract: The identification of the proteins that comprise the serum proteome is a current major research goal that will provide useful information for the diagnosis and treatment of various diseases. It is well established that Hsp70 and Hsp70 antibodies are present in human serum. This study reports on the development of an ELISA assay for the Hsp70 co-chaperone, HspBP1. HspBP1 is present in human serum at concentrations ranging between 0.74 to 3.98 ng/mL. No gender or age differences in the HspBP1 levels were identified. It was also found that human serum contained antibodies to HspBP1, and there were no gender or age differences in these levels. In addition, there was no correlation between the level of HspBP1 in a sample and the antibody titer. Finally, we found that HspBP1 in serum is complexed

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to anti-HspBP1 antibodies. This report provides initial baseline data on HspBP1 in human serum and provides the methods for future studies to determine if these levels are altered in response to disease.

Keywords: HspBP1, Co-chaperone, Hsp70, Serum

INTRODUCTION

Circulating blood proteins have the potential of profiling various disease states and therefore have tremendous diagnostic and treatment value. The identification and quantification of the proteins in human sera/plasma proteome has not been completed. A complete catalogue of these proteins is the goal of the Human Proteome Organization.[1] Serum proteins are present in a wide range of concentrations from mg/mL to pg/mL; therefore, it is unlikely that one technique will be able to detect all the proteins. Currently, the major emphasis is to use either two-dimensional electrophoresis or multidimensional liquid chromatography to separate proteins followed by identification of individual proteins by mass spectrometry. A major problem has been the identification and quantification of low abundance proteins. This is because a small number of proteins including albumin, immunoglobulins, α2-macroglobulin and transferrin make up approximately 80% of total serum proteins. An alternative approach is to focus on a single protein and determine its presence or absence using antibody and ELISA techniques. This method does not provide a total profile of the serum/plasma proteins, but does identify specific proteins, and this information could be used to verify the whole proteome approaches or design antibody arrays for proteome analysis. In addition, the whole proteome approach does not identify antibodies against individual proteins, yet characterization of these serum/plasma components may also have clinical use.

Heat stress proteins are a highly conserved set of proteins found in all eukaryotic cells. The most studied of these proteins has a molecular weight of approximately 70 kilodaltons and therefore is called Hsp70 (heat stress protein 70). The intracellular levels of Hsp70 increase in response to numerous environmental stresses including an increase in temperature, hypoxia and exposure to heavy metals. The increased levels of Hsp70 enhance protection and recovery from the stresses. Hsp70 is considered an intracellular protein but detectable amounts have been reported in the serum of normal individuals.[2]

This laboratory has identified a novel cochaperone regulator of Hsp70 called HspBP1.[3] HspBP1 binds to the ATPase domain of Hsp70 and inhibits the ability of Hsp70 to refold denatured proteins. HspBP1 blocks the ability of the ATPase domain to bind ATP and ADP and stimulates the removal of prebound ATP and ADP.[4] HspBP1 is abundant in tissues and the levels of both Hsp70 and HspBP1 are elevated in numerous tumors.[5] Recently, the structure of HspBP1 was reported with evidence that HspBP1

can act as a nucleotide exchange factor.[6] The fact that both Hsp70 and anti-Hsp70 antibodies are present in human sera prompted us to investigate if the Hsp70 cochaperone HspBP1 and antibodies against HspBP1 could also be detected in serum from healthy individuals.

EXPERIMENTAL

Human Subjects

Volunteers gave written consent for participation in this study as approved by the University of Arizona Institutional Review Board. The twenty subjects (10 males and 10 females) recruited for this research ranged in age from 18–56 years (mean age of 28.7). The sample population consisted of 80% white/caucasian, 15% hispanic and 5% other. Subjects were reportedly in general good health and had no history of chronic disease. Nonfasting specimens were collected into a 7 mL Royal Blue (BD, Franklin Lakes, NJ) tube with no additive using standard venipuncture technique. Tubes were allowed to clot in a vertical position for one hour before centrifugation for 10 minutes at 3,500 revolutions/min. Serum aliquots were then transferred to sterile polypropylene tubes and stored at -75°C within two hours of collection.

Proteins and Antibody Production in Sheep

HspBP1 preparations used in this report are either the truncated mutant that is lacking the first 83 amino acids (HspBP1 84-359) and is described in McLellan et al.[7] or the full length protein described in Raynes and Guerriero.[3] Both proteins reacted the same in all procedures. The procedures and protocol for HspBP1 antibody production in sheep were reviewed and approved by the University of Arizona Institutional Animal Use and Care Committee. Two one year old Rambouillet sheep were injected with 200 μg of HspBP1 in Freunds complete adjuvant (50 : 50 mixture). The sheep were boosted twice with 133 μg HspBP1 in Freund's incomplete adjuvant at two week intervals. Thereafter, the animals were boosted (50–100 μg HspBP1) every month and bled every two weeks (50–100 mL per sheep). Serum was stored at -20°C until used to purify antibodies. HspBP1 antibodies were purified from serum using a HspBP1 affinity column.

Immunoprecipitation of HspBP1 with Antibodies Coupled to Sepharose

HspBP1 antibodies were coupled to Sepharose as recommended by the manufacturer. Briefly, two mL of a slurry of NHS-activated Separose 4 Fast Flow

beads (GE Healthcare, Piscataway, NJ) were rinsed through filter paper with 20 mL of 1 mM HCl. One mL (1.22 mg/mL) of sheep anti-HspBP1 antibody (Novus Biologicals, Littleton, CO) in phosphate buffered saline, pH 7.4, was added to the beads and the pH was adjusted to 7.5 with 2M NaOH. The slurry was incubated while shaking for 3 hours at room temperature. The beads were then rinsed, on filter paper, with phosphate buffered saline and incubated with 2 mL of 1 M Tris-HCl (pH 8.0) for 2 hours at room temperature to block unreactive groups. The slurry was then poured into a column and rinsed three times with 1 mL of 0.1M Tris-HCl (pH 8.0) and then 3 times with 1 mL of 0.1M acetate (pH 3.5), with five repeats. The beads were stored in phosphate buffered saline containing 0.2% NaN_3.

The coupled beads (100 μL of slurry) were next added to 2.2 mL of human serum and incubated at room temperature with shaking for 2 hours. The beads were pelleted by centrifugation in a microfuge at 4,000 rpm and rinsed 4 times with 1 mL of PBS. Proteins were eluted from the pellet by the addition of 50 μL SDS sample buffer, heated for 3 min. at 100°C and centrifuged. Gels were run on 12.5% PAGE, transferred to nitrocellulose (Schleicher and Schuell, Keene, NH), and blocked with 5% milk in TBST (0.9% NaCl, 0.05% Tween-20 and 10 mM Tris pH 7.5). Membranes were incubated overnight at 4°C with 1 μg/mL anti-HspBP1 monoclonal antibody (cat. no. MAB-10201, Orbigen, San Diego, CA). The blots were washed with TBST and incubated for 1 hr at room temperature in horseradish peroxidase conjugated secondary antibody (Jackson Immuno Research Labs, Inc., West Grove, PA) at a concentration of 0.04 μg/mL. Following extensive washing in TBST, reactions were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Inc., Rockford, IL) using BioMax Light film (Eastman Kodak, Rochester, NY).

ELISA for HspBP1

Individual wells of a 96-well micotiter plate (Nunc-Immuno 96 MaxiSorp, NuncNalge International, Rochester, NY) were coated overnight at 4°C with 100 μL of 1 μg/mL sheep anti-HspBP1 antibody (Novus Biologicals, Littleton, CO) in phosphate buffered saline (PBS). Plates were sealed with plastic wrap during all incubations. Wells were rinsed 3 times with PBS + 0.05% Tween-20 and blotted on paper towel. Wells were blocked with 200 μL of 5% nonfat dry milk in PBS plus 0.05% Tween-20 for 1 hour at room temperature with shaking. Plates were rinsed 3 times with PBS plus 0.05% Tween and blotted dry.

Serum samples were diluted 1 : 5 and then serial diluted in blocking buffer (either with or without 1 mM PMSF and 1 mM benzamidine). 100 μL per well of standard curve samples contained HspBP1 (84-359), purified as described,[2] were serial diluted from 6.25 ng/mL-0.024 ng/mL or unknowns in blocking solution (with or without 1 mM PMSF and

1 mM benzamidine) were added in duplicate and incubated for 2 hours at room temperature with shaking. Wells were aspirated into a container with bleach. Plates were rinsed six times as above and blotted dry. Rabbit anti-HspBP1 (100 μL per well of 0.1 μg/mL) (Delta Biolabs, Campbell, CA) was added in blocking solution and incubated at room temperature for 1 hour with shaking. Plates were rinsed six times and blotted dry. Goat anti-rabbit IgG HRP conjugated antibody (Pierce, Rockford, IL), 100 μL per well of 1 : 5,000 dilution was added (already made 50% glycerol - final concentration 0.08 μg/mL) and the plate was incubated at room temperature for 1 hour with shaking. Plates were then rinsed 6 times, blotted dry, and 100 μL of TMB (Sigma T5559 Super Slow) was added per well. Plates were then incubated for 5–10 min at room temp with shaking, followed by the addition of 100 μL of 2M H_2SO_4 per well to stop the reaction. Absorbance was read at 450 nm using a microplate reader (Thermo Electron Corp. Multiscan MCC/340, Vantaa, Finland) a standard curve was plotted and unknowns were determined from the standard curve.

ELISA for Anti-HspBP1 Levels

Plates (96-well micotiter plates, Nunc-Immuno 96 MaxiSorp, NuncNalge International, Rochester, NY) were coated overnight at 4°C with 100 μL of 1 μg/mL HspBP1(84-359). Plates were sealed with plastic wrap during all incubations. Plates were rinsed three times with PBS + 0.05% Tween-20 and blotted on a paper towel. Blocking was done with 200 μL of 5% nonfat dry milk in PBS + 0.05% Tween for 1 hour at room temperature with shaking. Wells were rinsed 3 times with PBS + 0.05% Tween and blotted dry.

Serum samples were diluted 1 : 10, followed by serial dilutions in blocking buffer. Diluted serum samples (100 μL) were added to the duplicate wells and the plate was incubated for 1 hour at room temperature with shaking. The plates were rinsed 4 times as above and blotted dry. The second antibody (goat anti-human IgG, IgA & IgM HRP conjugated, Jackson Immunoresearch Lab., Inc.) was made 50% in glycerol to a concentration of 0.4 mg/mL and diluted 1 : 5,000 in blocking buffer for a final concentration of 0.08 μg/mL. Aliquots (100 μL) were added to each well and the plate was incubated at room temperature for 1 hour with shaking, followed by rinsing 4 times. Plates were developed by the addition of 100 μL ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid) per well and incubated for 30 min at room temperature with shaking. ABTS (Sigma-Aldrich, St. Louis, MO) solution was made by dissolving one 10 mg tablet in 100 mL 0.1M citrate buffer pH 4.2; 30% H_2O_2 was added to 0.03% immediately prior to use. Plates were read in a microplate reader at 414 nm.

RESULTS

Identification of HspBP1 in Human Serum

Initial attempts to identify HspBP1 in serum, using western blots of whole serum or serum depleted of albumin and immunoglobulins, were unsuccessful. Therefore, an alternative approach, using anti-HspBP1 antibodies linked to sepharose beads, was used so the HspPB1 in a larger amount of serum ($\sim$2 mL) could be analyzed. This approach resulted in isolation of a sufficient quantity of HspBP1 that could be detected on a western blot (Fig. 1). The purified recombinant HspBP1 (Fig. 1, lane 2) is approximately 2 kDa larger than the native protein, due to the presence of the His tag and accompanying amino acids. Sepharose beads without linked antibody did not pull down HspBP1 (results not shown).

Characterization of HspBP1 ELISA

The sensitivity of the ELISA for HspBP1 was determined by adding two standard deviations to the mean optical density value of 76 replicates of samples containing no HspBP1 and calculating the corresponding

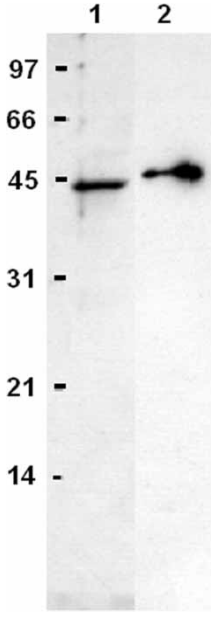

Figure 1. Analysis of HspBP1 in human serum. Human serum was incubated with anti-HspBP1 antibody beads, the bound proteins were eluted and analyzed by western blot (Lane 1) and compared to 25 ng of purified HspBP1 (Lane 2).

concentration. The minimum detectable dose is 6 pg/mL. The intra assay precision was determined by analyzing 20 samples, in duplicate, on one plate. These assays had a coefficient of variance of 4.6%. The inter assay precision was determined by analyzing 25 samples, in duplicate, on 5 different plates. These assays had a coefficient of variance of 8.8%. A typical standard curve with a range of 50 pg/mL to 3,000 pg/mL is presented in Fig. 2. Analysis of these data resulted in a correlation coefficient of 0.998 between optical density and concentration of HspBP1.

Levels of HspBP1 in Human Sera

Levels of HspBP1 were determined in sera collected from 20 adult volunteers, including 10 males and 10 females of variable ages. HspBP1 levels ranged from 0.74 ng/mL to 3.98 ng/mL (Fig. 3A). The results were analyzed for age (Fig. 3B) and gender (Fig. 3C) differences in HspBP1 levels and none were found. In addition, a comparison was made between blood allowed to coaglulate (serum) and not allowed to coagulate (plasma) from the same individual and no differences were found (data not shown).

Anti-HspBP1 Antibodies in Human Sera

The fact that human sera contained HspBP1 initiated a study to determine if the endogenous HspBP1 could invoke an immune response resulting in circulating levels of ant-HspBP1 antibodies. A typical dilution curve is shown in

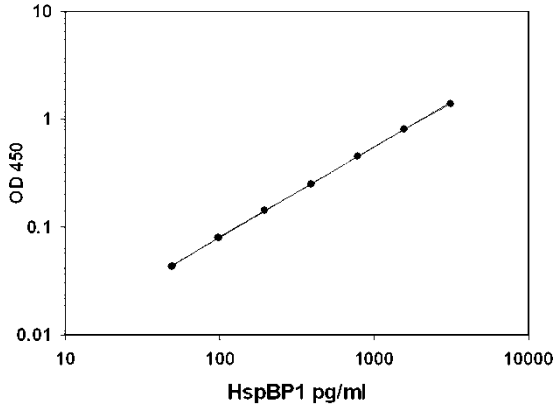

Figure 2. Standard curve for HspBP1 ELISA. The assay was performed as described in Experimental.

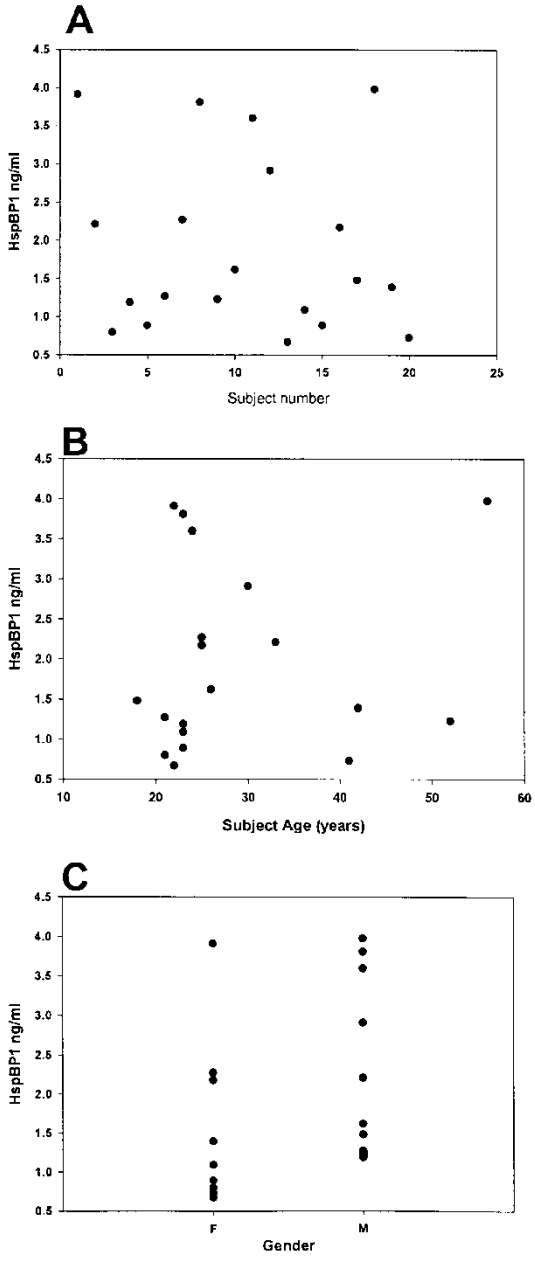

Figure 3. Levels of HspBP1 in human sera. Serum was collected from volunteers and analyzed for HspBP1 using the ELISA described in Experimental (Panel A). Levels of HspBP1 were plotted according to the ages of the volunteers (Panel B), and their gender (Panel C).

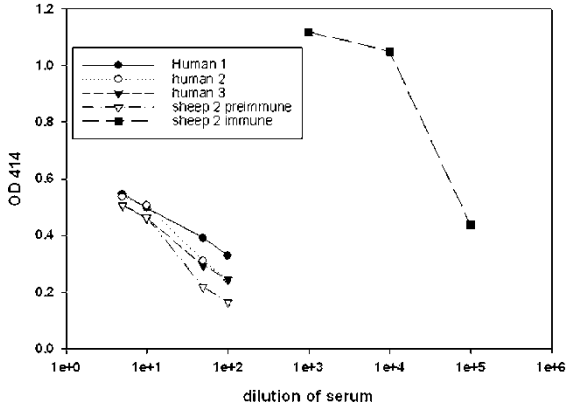

Figure 4. ELISA for antibodies in sera. Recombinant HspBP1 was first bound to the wells and sera was assayed as described in Experimental. Three human and two sheep samples were assayed. The sheep samples were both before and after immunization with HspBP1.

Fig. 4; three different human sera were serially diluted and assayed for HspBP1 antibodies. In addition, the serum from a pre-immune sheep and the serum from the same sheep immunized with HspBP1 were analyzed. The human subjects and non-immunized sheep have been shown to have similar antibody levels, whereas, the titer is the immunized sheep is approximately three orders of magnitude higher.

The 20 human samples used for the detection of HspBP1 also were analyzed for the presence of HspBP1 antibodies. The levels of HspBP1 antibody (O.D. 414 of 1 : 10 dilution) ranged from approximately 0.7 to 1.1 (Fig. 5A). Analysis of the data based on gender (Fig. 5B) or age (Fig. 5C) did not indicate any trends. In addition, the data were analyzed for a correlation between HspBP1 and HspBP1 antibody levels in the matched samples and no significant correlation was found (Fig. 5D).

HspBP1 Bound to HspBP1 Antibodies in Serum

The finding that human sera contained both HspBP1 protein and anti-HspBP1 antibodies led to the speculation that HspBP1 in serum could be bound to HspBP1 antibodies. Experiments were conducted to test this possibility by coating ELISA plates with either HspBP1 antibody or HspBP1 protein and incubating with human serum. Wells coated with HspBP1 antibody would bind serum HspBP1 and any associated binding proteins, whereas wells coated with HspBP1 would bind serum HspBP1 antibodies. The plates were developed by incubating with an anti-human IgG conjugated antibody.

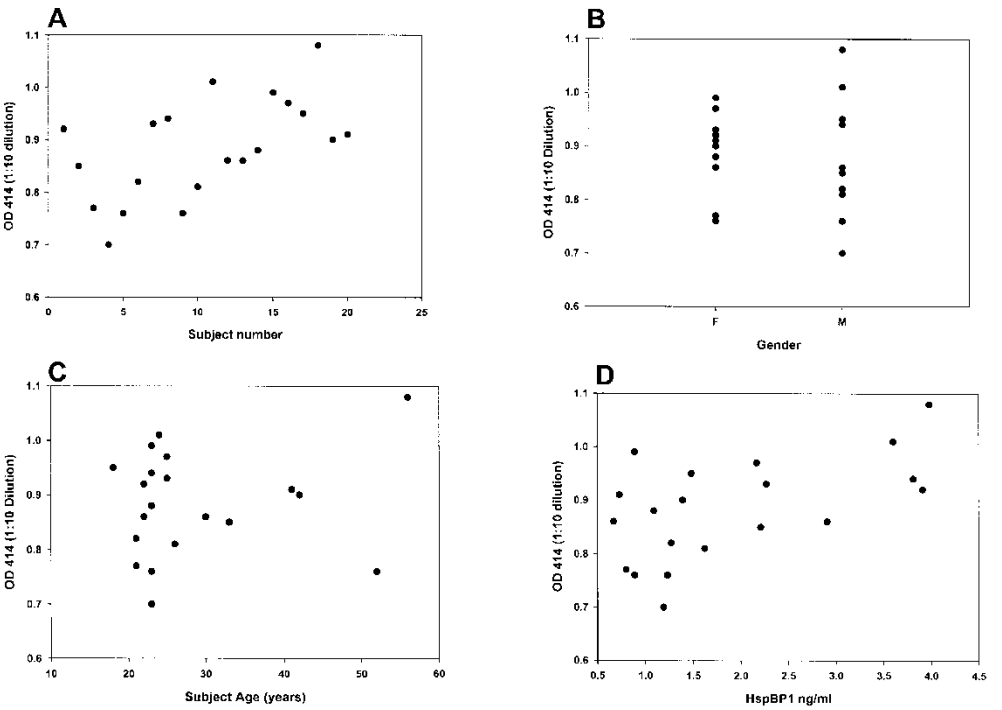

Figure 5. Levels of HspBP1 antibody in human sera. The identical serum samples analyzed in Fig. 3 were assayed for anti-HspBP1 antibodies as described in Experimental (Panel A). Antibody titers were also analyzed for gender (Panel B), and age (Panel C) differences. Panel D is a plot of HspBP1 level and corresponding HspBP1 antibody titers for each of the 20 samples.

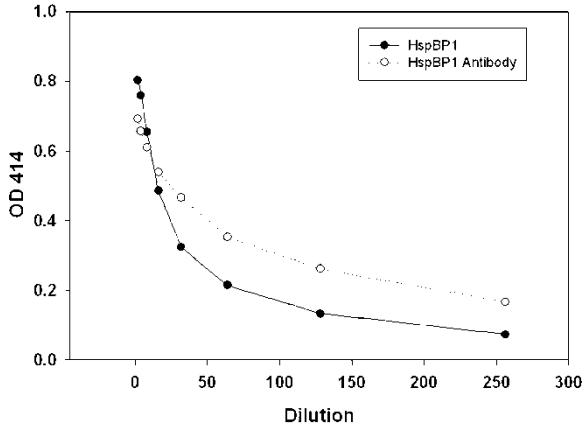

Figure 6. Detection of HspBP1 antibodies bound to serum HspBP1. Plates were coated with either sheep anti-HspBP1 or recombinant HspBP1 protein and incubated with the indicated dilution of human serum. Backgrounds were subtracted from each point and consisted of wells that were not coated with antibody or protein. Plates were incubated with anti-human IgG, IgA, IgM to detect human antibodies and processed as described in Experimental.

Wells coated with sheep anti-HspBP1 were positive for human IgG, IgA and IgM, indicating that the serum HspBP1 that bound to the wells had human anti-HspBP1 bound to it (Figure 6).

DISCUSSION

The Hsp70 cochaperone HspBP1 and antibodies against HspBP1 are found in human serum. The measured levels of Hsp70 found in normal serum/plasma have been shown, in other research, to vary between <5 ng/mL and 3,000 ng/mL.[8–11] This is a $>3{,}000$-fold difference across studies and it is not known if this wide variation is real or is due to differences in the assays used. The levels of HspBP1 reported here (0.74 to 3.98 ng/mL) from human serum samples are similar to the lower end of the serum Hsp70 levels and, therefore, suggest that HspBP1 might be regulating serum Hsp70. Extracellular Hsp70 can stimulate the immune response, either by binding to immunogenic peptides or by induction of cytokines.[12,13] One possibility is that HspBP1 regulates the immune response by binding and regulating the Hsp70.

Serum Hsp70 levels can be altered under numerous conditions, such as inflammatory status,[10,14] postoperative organ dysfunction,[8] age,[11,15] risk of coronary artery disease,[16] survival after trauma,[17] chronic heart failure,[18] longevity propensity,[9] and prostate cancer.[19] In addition, circulating antibodies against Hsp70 are elevated in patients with various conditions.[20] Oka et al.[21] reported that lung tumors contained elevated levels

of the Hsp70 cochaperone Hsp40 and the sera from the same lung cancer patients contained elevated levels of Hsp40 antibodies. These findings suggest that the immune system recognizes these proteins when they are present in the circulatory system. The presence of HspBP1 protein and antibodies in human sera are consistent with this idea.

A novel finding reported here is that the serum HspBP1 is associated with anti-HspBP1 antibodies. This raises the question that, if these serum proteins are proposed to be biologically active, what percentage of the circulating proteins are bound to antibodies and are thereby rendered non functional. It should also be pointed out that such an interaction would not be detectable using other techniques for characterization of the serum proteome, such as mass spectrometry.

The relatively narrow range of circulating HspBP1 found in our samples from healthy adults suggests that, if the levels of HspBP1 are altered in some disease state, it might be possible to monitor serum HspBP1 for diagnostic evaluation. Recently, it was reported that HspBP1 levels are elevated in some tumors.[5] It is possible that cancer patients may have elevated serum HspBP1 levels due to the protein being released from tumor cells. Future research will focus on monitoring Hsp70 and Hsp70 antibody levels in diseased states.

ABBREVIATION

HspBP1, heat shock protein binding protein 1.

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