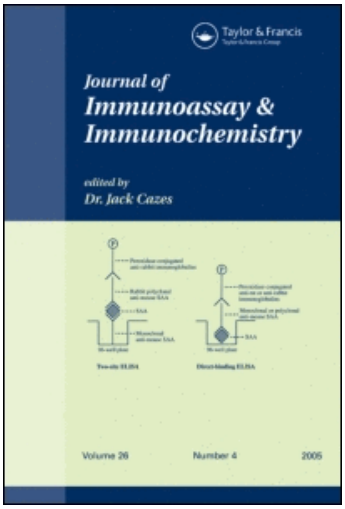

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Detection of the Soluble Heat Shock Protein 27 (hsp27) in Human Serum by an ELISA

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ABSTRACT

Increased levels of autoantibodies against heat shock protein 27 (hsp27) in patients with breast, ovarian, or endometrial cancer strongly suggest the presence and increased levels of hsp27 in their circulation. Therefore, we have developed a sensitive and reproducible ELISA for quantification of soluble hsp27 levels in biological fluid such as serum. The assay is highly specific for hsp27. The limit of detection of the ELISA is about 0.5 ng/mL. The mean intra- and inter-coefficients of variation were 7.45 and 8.18, respectively. The recovery of the recombinant protein was nearly 100%. The assay could detect soluble hsp27 levels in normal human serum when the level was >0.5 ng/mL. Out of 28 serum samples we tested, 10 samples were not detected for any hsp27 level in our ELISA. However, hsp27 levels could be detected in the other 18 samples. The median serum hsp27 level was 3.27 ng/mL when all the 28 normal control samples were included. Low levels of hsp27 in normal human serum may be useful to distinguish the

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hsp27 levels in breast or other cancer patients during the progression of the disease. Therefore, the use of hsp27 ELISA could be extremely useful in evaluating the role of soluble hsp27 in breast or other cancers.

Key Words: Human serum; hsp27; ELISA; Cancer tissues.

INTRODUCTION

Heat shock proteins (hsp), stress response proteins found in all species, are thought to play a protective role in cells under stress.[1,2] Many of the hsps are also constitutively expressed, playing important roles as molecular chaperones in normal cell functions.[2] We are particularly interested in one of the small hsps, hsp27, because this protein appears with increased frequency in human breast cancer tissues.[3,4] Increased expression of hsp27 in breast cancer cells has long been known to increase resistance to chemotherapeutic drugs.[4,5] Moreover, hsp27 overexpression has been shown to be associated with shorter disease-free survival in patients with breast cancer.[3,6,7] Antibodies against hsp27 have been detected in the sera from patients with breast and gynecological cancer.[8–10] These antibody data strongly suggest the presence of hsp27 protein in the circulation. Recently, we have demonstrated that exogenous addition of recombinant hsp27 to human monocytes induces production of interleukin-10 (IL-10) and tumor necrosis factor-α (TNFα), indicating the possible effect of extracellular hsp27 on immune cells.[11] Fanelli et al. could detect serum hsp27 levels in breast cancer patients as well as in normal control groups.[12] Although the mean hsp27 levels in cancer patients were higher than in control subjects, hsp27 levels did not have significant correlation with the progression of the disease.[12] However, as described, hsp27 levels in the serum could not be detected by Western blotting.[12] Instead, hsp27 in the serum could only be sensitively detected when immunoprecipitation of hsp27 by anti-hsp27 antibody followed by immunoblotting of the immunoprecipitated protein was performed.[12] Such lengthy assays are often too complex and are not too quantitative. Recently, cell-free hsp27 has been detected in the lower genital tract of women with ovarian or endometrial cancer by an ELISA using cytochrome *c* bound to microtiter plate wells,[13] based on the report that hsp27 can bind to cytochrome *c* in vivo.[14,15] In such methods, only the hsp27 molecules that can bind to cytochrome *c* could be detected. Thus, the role of cell-free hsp27 levels in breast or gynecological cancer should be reevaluated using other simple, quantitative and accurate method such as ELISA using anti-hsp27 antibody as capture antibody for highly specific binding. Therefore, we have developed a simple ELISA method using anti-hsp27 monoclonal antibody for detection of hsp27 in biological fluids such as serum.

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EXPERIMENTAL

Materials

High binding, flat bottom, 96 well polystyrene EIA/RIA plates were obtained from Corning Incorporated (Corning, NY). Human recombinant hsp27 (hsp27, product number—SPP715), human recombinant hsp70 (product number—SPP755) and the mouse monoclonal antibody against human hsp27 (product number—SPA-800) were purchased from Stressgen Biotechnologies (Victoria, Canada). Rabbit polyclonal antibody against human hsp27 (Catalogue number—sc9012) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit polyclonal antibody was obtained from Cell Signaling Technology (Beverly, MA). Protease-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). The color reagents—hydrogen peroxide and tetramethyl benzidine (TMB) were purchased from BD Pharmingen (San Diego, CA). Recombinant cytokines such as interleukin (IL)-4, TNFα, interferon-γ (IFNγ), and granulocyte-macrophage colony stimulating factor (GM-CSF) were purchased from Peprotech Inc. (Rocky Hill, NJ).

Blood was collected from 28 normal volunteers (21 women and 7 men) for isolation of serum. Their ages ranged from 20 to 50. Informed consent was obtained from every volunteer before the blood draw and the study was approved by the Institutional Review Board. Five to six milliliters of peripheral blood was allowed to clot at room temperature for serum collection. After 4–5 hr, clotted blood was centrifuged at 1200*g* for 10 min at 4°C. Serum fractions were carefully isolated and centrifuged another time to exclude the possibility of any cell contamination. Serum samples were kept frozen at −80°C until the day of assay for hsp27.

Preparation of Plates

Microtiter plates were coated with 100 μL of solution of mouse anti-human hsp27 MAb (4 μg/mL) in 10 mM phosphate-buffered saline (PBS, Mediatech Inc., Herndon, VA). Plates were sealed with ELISA plate sealer and incubated at room temperature overnight and then washed (by filling up the wells) four times with wash buffer (10 mM PBS containing 0.05% Tween-20). All wells were then blocked with 300 μL of blocking buffer (1% BSA in PBS containing 0.05% Tween-20) by incubating the plate for 1 hr at room temperature on a plate shaker (about 400 rpm).

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ELISA Procedure

The plates were washed four times with wash buffer. One hundred microliters of blocking buffer is added to every well. Human recombinant hsp27 was diluted serially in blocking buffer and then 100 μL of 0.94, 1.875, 3.75, 7.5, 15, 30, and 60 ng/mL was added to generate a standard curve. One hundred microliters of undiluted or diluted (1 : 2, 1 : 5 in blocking buffer) serum was added per well. One hundred microliters of blocking buffer alone was added to wells designated as blank for subtraction of background color. The plates were sealed with ELISA plate sealer and incubated for 2 hr at room temperature on a plate shaker (about 400 rpm). The plates were then washed four times with wash buffer. To each well, 100 μL of rabbit anti-human hsp27 (250 ng/mL, diluted in blocking buffer) was added, the plates were sealed with ELISA plate sealer and incubated for 1 hr at room temperature on a plate shaker (about 400 rpm), washed four times with wash buffer. To each well, 100 μL of goat anti-rabbit polyclonal antibody conjugated to HRP (1 : 5000 in blocking buffer) was added to each well, the plates were sealed with ELISA plate sealer and then incubated for 1 hr at room temperature on a plate shaker (about 400 rpm). After four washes with wash buffer, 100 μL of color reagent (freshly made equal mixture of TMB and hydrogen peroxide) was added to each well and the plate was incubated uncovered for 30 min in the dark at room temperature on a shaker (about 400 rpm). The reaction was stopped with 100 μL/well of 2 N sulfuric acid. The absorbance at 450 nm was obtained on an ELISA microplate reader with a reference wavelength of 550 nm. The values from the blank wells (background color absorbance) were subtracted from the values of standard or sample wells to obtain hsp27 bound specific absorbance. From the standard curve, the hsp27 levels in each test sample were quantitated.

Data Analysis

Data are expressed as mean ± SEM or median as appropriate. Linear regression analysis was performed for evaluation of linearity. Coefficient of variation (CV) was used for assessment of intra- and inter-assay variation.

RESULTS

Assay Optimization

A number of parameters were tested in order to optimize the assay. These included the concentration of mouse anti-human hsp27 MAb for coating,

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concentration of rabbit anti-human hsp27 PAb, concentrations of HRP-bound goat anti-rabbit antibody, deciding the concentrations/dilutions of recombinant human hsp27 for obtaining the linear portion of the standard curve and the serum dilutions. The coating concentration of mouse anti-human hsp27 MAb was tested by coating plates with 100 μL of 0.01, 0.5, 1, 2, 4, 8, and 10 μg/mL solutions for detection of different concentrations of recombinant hsp27. The optimum concentration was found to be 4 μg/mL. The rabbit anti-human hsp27 PAb was used at different concentrations, e.g., 10, 50, 100, 250, 500, and 1000 ng/mL for titration and the optimum concentration was determined to be 250 ng/mL. Finally, the concentration of HRP-bound goat anti-rabbit antibody was titrated to be 1 : 5000 (1 : 500, 1 : 1000, 1 : 2000, 1 : 5000, 1 : 10000, and 1 : 20000 were tested). For optimization of standard curve, the recombinant human hsp27 protein was serially diluted (1 : 2) with the highest concentration starting from 500 ng/mL. Figure 1A shows the standard curve of absorbance at 450 nm against the concentration of hsp27 protein (ranging from 500 to 0.976 ng/mL). The last seven concentrations ranging from 62.5 to 0.976 ng/mL exhibited the most linear nature of the standard curve (Fig. 1B). Finally, the serum samples were tested at different dilutions. However, because of the very low concentration of hsp27 levels (data shown later) in serum samples from normal human volunteers, samples were mostly used undiluted or diluted only 1 : 2.

Specificity

Several proteins were used to test the specificity of the assay. We used a number of cytokines such as TNFα, IFNγ, GM-CSF and IL-4 at two different concentrations (10 and 100 ng/mL). All of these cytokines did not show any cross-reactivity with this ELISA at any of the above two concentrations in two different assays. We also tested insulin, another non-related protein, at the concentration of 100 ng/mL. Our ELISA did not have any cross-reactivity with insulin even at this elevated concentration. Finally, we tested whether the ELISA for hsp27 had any cross-reactivity with other hsp such as hsp70. Hsp70 was used at the concentrations of 10 and 100 ng/mL. Our ELISA did not show any cross-reactivity with hsp70 at any of the above concentrations.

Sensitivity

The standard curve was quite linear up to the concentration of 62.5 ng/mL, exhibiting very high sensitivity up to this concentration (Fig. 1A and B). Our ELISA could even detect up to 125 ng/mL with 85.7 $\pm$ 2.67% ($n = 6$)

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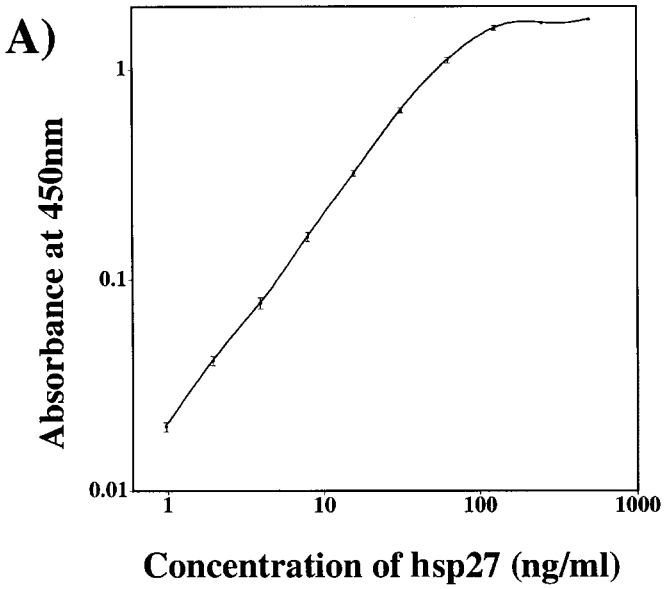

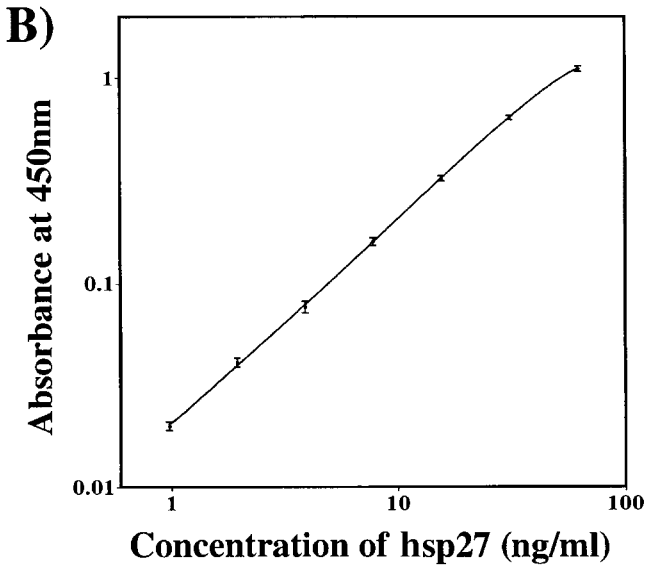

Figure 1. hsp27 standard curve. (A) Recombinant human hsp27 protein was serially diluted (1 : 2) with the highest concentration starting from 500 ng/mL. The absorbance at 450 nm is plotted (log–log) against the concentration of hsp27 protein. (B) The absorbance at 450 nm is plotted against recombinant hsp27 ranging only from 62.5 to 0.976 ng/mL.

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accuracy from two independent assays each with triplicate runs. Towards the lower range, our ELISA could detect up to 0.5 ng/mL that is similar to the one recently introduced by Oncogene Research Products (lower range up to 0.78 ng/mL).

Assay Performance and Application

Recovery of Recombinant hsp27

Recombinant hsp27 was used at the concentration of 25 ng/mL in seven different wells. ELISA was performed along with a standard curve (concentration range of 60–0.94 ng/mL). The values obtained from the seven replicates were 24.3, 28.48, 26.61, 24.6, 26.03, 24.12, and 23.24 with the mean ± SEM of 25.33 ± 0.68. Thus, the % of recovery was near 100%.

Precision (Intra- and Inter-assay)

To test the assay precision, we had used three human serum samples that were tested before by our ELISA and known to have high, medium and low titers of hsp27 levels. The samples were run in quadruplicate in three different plates. Hsp27 levels were calculated from respective standard curves. Tables 1 and 2 indicate the intra- and inter-assay precision respectively [as expressed by a % CV] obtained from these three serum samples. The mean intra- and inter-assay % CV from these three samples were 7.45 and 8.18, respectively. The CV under 10% shows the precise and reproducible aspects of our ELISA.[16]

hsp27 Levels in Human Serum

To test the utility of the assay, we measured the hsp27 levels in serum samples isolated from 28 normal healthy volunteers. Out of 28 serum samples

Table 1. Intra-assay precision of serum hsp27 levels.

	Mean hsp27 levels ± SEM (ng/mL)	CV (%)
Sample 1	3.83 ± 0.22	10.006
Sample 2	37.09 ± 1.19	6.45
Sample 3	49.89 ± 1.45	5.83

Note: $n = 4$.

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Table 2. Inter-assay precision of serum hsp27 levels.

	Mean hsp27 levels ± SEM (ng/mL)	CV (%)
Sample 1	3.57 ± 0.23	11.2
Sample 2	35.92 ± 1.55	7.49
Sample 3	49.21 ± 1.66	5.87

Note: $n = 3$.

we tested, hsp27 could not be detected in 10 samples. These samples may still contain some hsp27 that is below our ELISA detection limit (~0.5 ng/mL). However, hsp27 levels could be detected in the other 18 samples we tested (Fig. 2). The median hsp27 level of all the 28 samples was 3.27 ng/mL (mean ± SEM = 9.8 ± 2.39) (Fig. 2). The data ranged from <0.5 ng/mL to 43 ng/mL. These data represent a wide variation in serum hsp27 levels among normal human subjects.

DISCUSSION

The major goal of this study was to develop a simple, quantitative, and accurate method, namely ELISA, for detection of hsp27 levels in biological fluids. Although the role of different hsps in chaperoning several polypeptides has long been known, their effects in immune functions such as cytokine production have only recently been established.[11,17,18] Detection of increased levels of autoantibodies against hsp27 in patients with breast or gynecological cancer strongly suggests the evaluation of any released hsp27 levels in circulation.[8–10] Therefore, we have developed an ELISA for detection of hsp27 levels in biological fluids such as serum.

Our hsp27 ELISA has the sensitivity up to 0.5 ng/mL. The sensitivity and the detection range of our hsp27 ELISA are similar to the commercially available hsp27 ELISA kit recently introduced by Oncogene Research Products. Because of the low levels of serum hsp27 levels in normal controls, our hsp27 ELISA could not detect soluble hsp27 in all of the normal serum samples tested. However, this ELISA kit could be sensitive enough to assess the difference in hsp27 levels in biological fluids from patients with breast or other cancers or any other disease when compared to healthy normal controls or a different subset of patients.

There is no report on secretion of hsp27 by any type of cell. Hsp27 is usually found in the cytoplasm of the cells. However, when MCF-7 cells are grown in ascites fluid of nude mice, hsp27 is found in the apical cytoplasm

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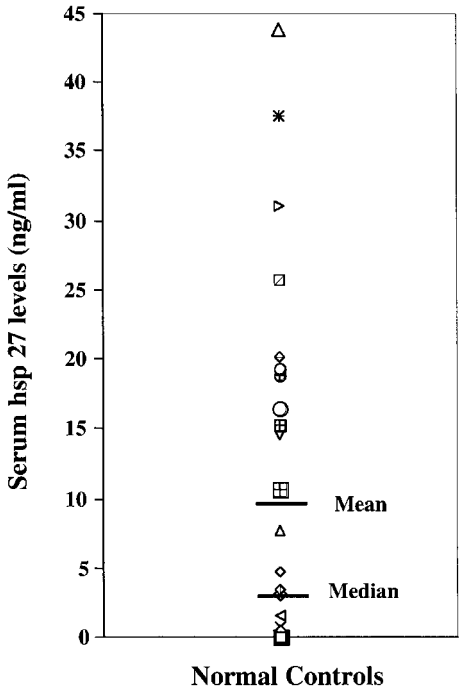

Figure 2. Detection of hsp27 in human serum. Serum samples from 28 normal healthy volunteers were assayed for soluble hsp27 levels in our ELISA. The data are represented both as mean as well as median.

of the cells, sometimes appearing in granules, suggesting its secretion.[19] Hsp27 could also be released from necrotic cells.[20] As mentioned in the introduction, presence of cell-free hsp27 has been detected in patients with breast or gynecological cancers.[12,13] We were able to detect hsp27 levels in the sera from some healthy donors when the hsp27 concentrations were $\geq 0.5\,\text{ng/mL}$. The wide variation in serum hsp27 levels among the healthy donors could be due to the known genetic variation in humans. Alternatively, the wide variation could originate from the difference in the extent of in vivo cell necrosis resulting from some low grade bacterial or viral infections of which our normal blood donors were not aware.

In conclusion, we have developed a sensitive and specific ELISA for quantitation of hsp27 in biological fluid. This method will help determine whether the secreted hsp27 can be used as biomarker for assessing the progression of the disease in breast or gynecological cancers, in particular, endometrial or ovarian cancer.

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ABBREVIATIONS

ELISA	Enzyme linked immunosorbent assay
hsp27	heat shock protein 27
PBS	phosphate buffered saline
BSA	bovine serum albumin
HRP	horseradish peroxidase
Mab	monoclonal antibody
Pab	polyclonal antibody

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