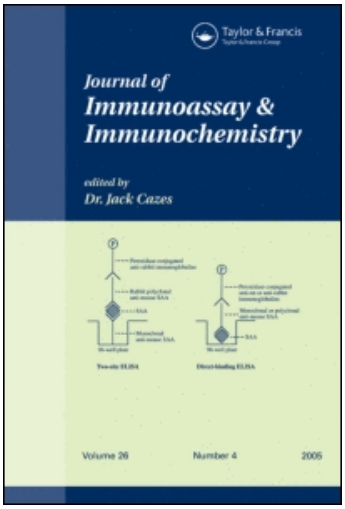

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Asit K. De[a]; Jennifer Strickland[a]
[a] Department of Surgery, University of Rochester Medical Center Rochester, NY, USA

To cite this Article De, Asit K. and Strickland, Jennifer(2007) 'Sensitivity of Flow Cytometric Assay for Measurement of Human Intracellular Heat Shock Protein 27', Journal of Immunoassay and Immunochemistry, 28: 3, 189 — 198
To link to this Article: DOI: 10.1080/15321810701454599
URL: http://dx.doi.org/10.1080/15321810701454599

Journal of Immunoassay & Immunochemistry, 28: 189–198, 2007

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810701454599

Sensitivity of Flow Cytometric Assay for Measurement of Human Intracellular Heat Shock Protein 27

Asit K. De and Jennifer Strickland
Department of Surgery, University of Rochester Medical Center
Rochester, NY, USA

Abstract: Increased expression of heat shock protein 27 (hsp27) is related to enhanced resistance of breast tumor cells to cytotoxic drugs and radiation therapy. Therefore, development of a rapid and sensitive method for detection of hsp27 may be useful for correlating tumor cell expression of hsp27 to breast cancer patients' clinical outcome. We have simultaneously assessed hsp27 levels in three different human cell lines (MCF-7, MDA-MB-231, and Jurkat) by both Western blotting and flow cytometry. MCF-7 hsp27 levels were consistently detected at higher levels, while MDA-MB-231 hsp27 levels were detected at very low levels when immunoblotting was performed. Hsp27 was not detected in Jurkat cells by immunoblotting. In contrast, hsp27 levels were detected by flow cytometry in all the cell lines, indicating a better sensitivity of this method. Although hsp27 was expressed in almost equal percentage of MCF-7 (93 $\pm$ 3.4%), MDA-MB-231 (97 $\pm$ 1%), and Jurkat (95.5 $\pm$ 1.9) cells, the fluorescence intensity of intracellular hsp27 protein was significantly lower in MDA-MB-231 and Jurkat cells as compared to MCF-7 cells. The flow cytometry data further demonstrated that reduced hsp27 expression in both MDA-MB-231 and Jurkat cells was not due to a lack of hsp27 expression in a subset of cells, but rather due to reduced expression of hsp27 in all individual cells.

Keywords: Human, Breast cancer, Hsp27, Flow cytometry, Western blotting, Sensitivity

Address correspondence to Asit K. De Ph.D., Department of Surgery, University of Rochester Medical Center Rochester, NY 14642, USA. E-mail: asit_de@urmc.rochester.edu

INTRODUCTION

Heat shock protein 27 (hsp27), an important member of the small hsp family, plays an important role as a molecular chaperone maintaining normal cell functions.[1,2] Hsp27 is constitutively expressed in different cell types and exerts its protective role in cells under stress due to its ability to inhibit apoptotic pathways.[1–4] Hsp27 expression is increased in breast and other cancer cells, and the increased expression of hsp27 can confer resistance to cytotoxic drugs, radiation therapy, and chemotherapy induced apoptosis.[5–9] Although there are several reports demonstrating a significant correlation of increased hsp27 expression in breast tumor tissues to poor clinical outcomes, there also exist reports suggesting no correlation between hsp27 expressions in breast tumor tissues and patients' clinical outcomes.[9–14] Many of these reports are on the basis of measurement of hsp27 in tumor sections by immunohistochemistry or in tumor cell lysates by Western blotting (immunoblotting). However, there are several consistent reports that induction of increased hsp27 expression in isolated breast and other tumor cell lines results in significantly increased inhibition of their apoptosis induced by chemotherapeutic drugs, radiation, or other apoptotic stimuli.[5,6,8,15] Assessment of hsp27 levels in isolated tumor cells may therefore provide more consistent data if hsp27 expression in isolated tumor cells is correlated to patients' responses to different therapies and their overall survival. Consequently, a method based on rapid detection with high sensitivity would be extremely beneficial for measurement of hsp27 in isolated tumor cells for studies correlating tumor hsp27 levels to breast cancer patients' clinical outcomes.

The conventional method for measurement of hsp27 levels in isolated cells (once cell lysates are prepared) is Western blotting. Recently, some commercially manufactured ELISA kits became available for measurement of hsp27 in cell lysates. We have also developed an ELISA for hsp27 measurement in human serum and the same assay can also measure hsp27 in cell lysates.[16] However, the sensitivity of these ELISA methods is similar to that of the immunoblotting method. In this study, we have chosen 3 different cell lines (MCF-7, MDA-MB-231, and Jurkat) with varying amounts of hsp27 expression and simultaneously assessed the expression of hsp27 both by immunoblotting and flow cytometry. Comparable levels of hsp27 were detected with both techniques in MCF-7 cells. Hsp27 levels in MDA-MB-231 cells were detected with low sensitivity by immunoblotting but with high sensitivity by flow cytometry. The presence of hsp27 in Jurkat cells was detected by flow cytometry with a high sensitivity, but was not detected by Western blotting at all, indicating a higher level of sensitivity of flow cytometric method for measurement of intracellular hsp27.

EXPERIMENTAL

Reagents

Human recombinant hsp27 and Fluorescence isothiocyanate (FITC) — conjugated mouse monoclonal antibody against human hsp27 (clone: G3.1) were purchased from Nventa Biopharmaceuticals Corp. (formerly Stressgen Biotechnologies) (Victoria, Canada). FITC conjugated isotype control (clone: MOPC-21), Cytofix/Cytoperm and Perm Wash reagents were purchased from BD Pharmingen (San Diego, CA). Rabbit polyclonal antibody against human hsp27 (H-77) and the goat polyclonal antibody against human α-actin (C-11 or I-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated molecular weight marker and horseradish peroxidase (HRP) conjugated goat anti-rabbit polyclonal antibody and ECL (enhanced chemiluminescence) reagents were obtained from Cell Signaling Technology (Beverly, MA). Protease-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Tris-glycine gels with a 4–20% gradient and nitrocellulose membranes were purchased from Biorad (Richmond, CA). A 100X protease inhibitor cocktail was purchased from Calbiochem (San Diego, CA). Hsp27 ELISA kit was purchased from Oncogene Research Products (San Diego, CA).

Maintenance of Cell Lines

The breast cancer cell lines, MCF-7 and MDA-MB-231, and the T-cell leukemic cell line, Jurkat, were purchased from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 was maintained in MEM (ATCC, Manassas, VA) medium, where as MDA-MB-231 and Jurkat were cultured in RPMI 1640 (Cellgro, Herndon, VA) medium, each medium was supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 50 U/mL penicillin G, 50 μg/ml streptomycin, 50 μg/mL gentamycin, 2.5 μg/ml fungizone, 4 mmol/L L-glutamine and 1% of MEM nonessential amino acids (Complete Medium). The cell lines were passaged twice a week.

Western Blot (Immunoblot) Analysis

Cell (1×10^6 of each type) lysates were prepared by addition of modified RIPA lysis buffer [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate and 1X protease inhibitor cocktail]. Equal amounts of protein (25 μg/lane) were subjected to 4–20% gradient Tris-glycine gel electrophoresis, transferred onto a nitrocellulose membrane and then blocked with 2% BSA-TBS buffer containing 0.01% Tween 20. Blots were then incubated with

anti-hsp27 polyclonal antibody (1:800 dilution), washed and then reacted with horseradish peroxidase (HRP) –conjugated secondary antibodies. The blots were developed by treatment with ECL reagents followed by analysis in a Hitachi CCDBIO 16SC imaging system using the Gene Tool software. The blots were stripped of the anti-hsp27 antibody by incubation of the membrane for 30 minutes at 50°C in a specific buffer (2% SDS, 100 mM 2-ME, 62.5 mM Tris-HCl, in dH_2O, pH 6.7) followed by re-probing with α-actin antibody for correction of loading differences.[17]

Flow Cytometry

Cells (2×10^5 of each type) were first fixed and permeabilized by treating the cells with 200 μL of Cytofix/Cytoperm reagent for 20 minutes at 4°C. Cells were washed twice with 2 mL of 1X Perm Wash solution. A total of 1×10^5 cells (two sets) were first treated with 1 μg of human IgG for 20 minutes followed by incubation with either 100 ng of FITC conjugated anti-hsp27 mAb (first set) or isotype control (second set), for 30 min at 4°C in the dark. Cells were washed twice with 2 mL of 1X Perm Wash and then resuspended in PBS containing 2% FBS and 0.05% sodium azide. Cells were then analyzed on a BD FACS Calibur flow cytometer. The antibody specificity was tested by separate incubation of the antibody (for 30 min) with 10-fold higher concentration of human recombinant hsp27 before its addition to the cells. Data for individual cells expressing hsp27 are presented as mean of percent positive cells as calculated by subtraction of the isotype control value from the hsp27 antibody value. Similarly, the data for overall intracellullar hsp27 expression are expressed as net mean fluorescence intensity (MFI) by subtraction of the isotype data from the hsp27 antibody data.

Data Analysis

Data are expressed as mean $\pm$ SEM. Student's t-test was performed to compare between the two data sets using Statview software. Statistical significance was considered when the p-value was < 0.05.

RESULTS

Comparison of Intracellular Expression of hsp27 in MCF-7, MDA-MB-231, and Jurkat Cells

We have simultaneously assessed intracellular expression of hsp27 by both the conventional method (Western blotting) and by flow cytometry in MCF-7, MDA-MB-231 (both human breast cancer cell lines), and Jurkat (a human

leukemic T cell line) cells. Figures 1A & 1B shows Western blotting data of one representative experiment whereas Figure 1C depicts the mean ± SEM of all 5 experiments performed. MCF-7 expression of hsp27 was sensitively detected (high band intensity) by Western blotting in all 5 experiments, whereas MDA-MB-231 expression of hsp27 was detected with very low band intensity (2 out of 5 experiments had extremely faint bands). As can be seen in Figure 1C, MCF-7 cells expressed significantly ($p = 0.002$; 19

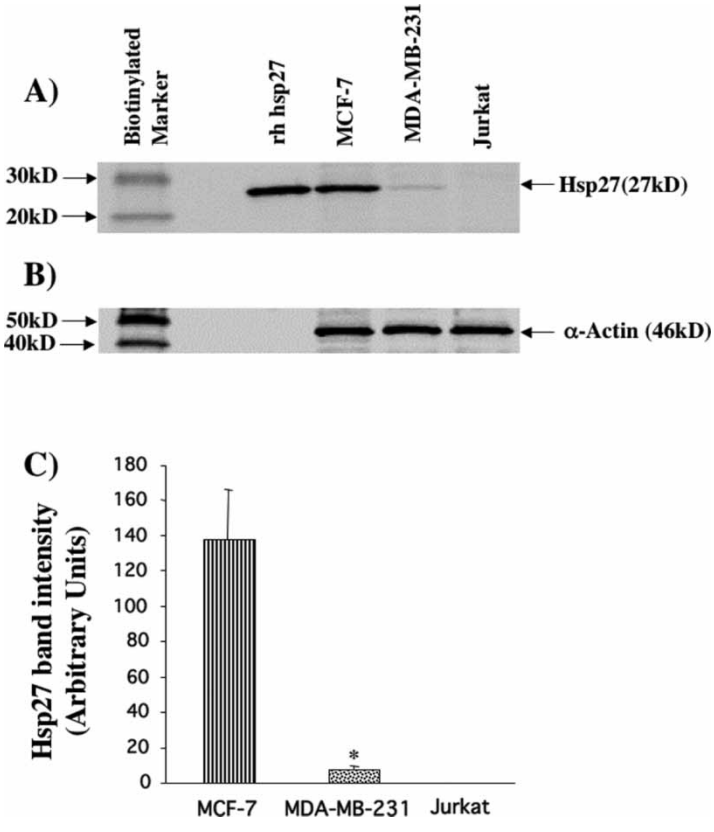

Figure 1. Western blotting analysis of hsp27 expression in MCF-7, MDA-MB-231 and Jurkat cells. A) Twenty-five micrograms of MCF-7, MDA-MB-231 and Jurkat cell lysates were subjected to 4–20% gradient gel electrophoresis and probed with anti-hsp27 antibody. Fifty nanogram of recombinant human hsp27 (rh hsp27) was used as a positive control. Biotinylated molecular weight markers were used for confirmation of molecular weights. Representative of 5 experiments. B) Blots were stripped and re-probed with anti-α-actin antibody for adjustment of any loading differences. C) Comparison of mean band intensity (already corrected for loading differences by α-actin expression) of hsp27 protein between MCF-7, MDA-MB-231 and Jurkat cells [*$p = 0.002$ (as compared to MCF-7 cells); $n = 5$].

fold) higher levels of hsp27 than MDA-MB-231 cells, as assessed by Western blotting and corrected by α-actin band intensity for loading differences. Surprisingly, hsp27 levels in Jurkat cells, despite similar α-actin band intensity, were too low to be detected by Western blotting in any of the 5 experiments.

In contrast to the Western blotting data, intracellular hsp27 levels were sensitively detected in all three cell lines by flow cytometry (Figure 2A-C). Surprisingly, even if the MCF-7 cells expressed significantly higher levels

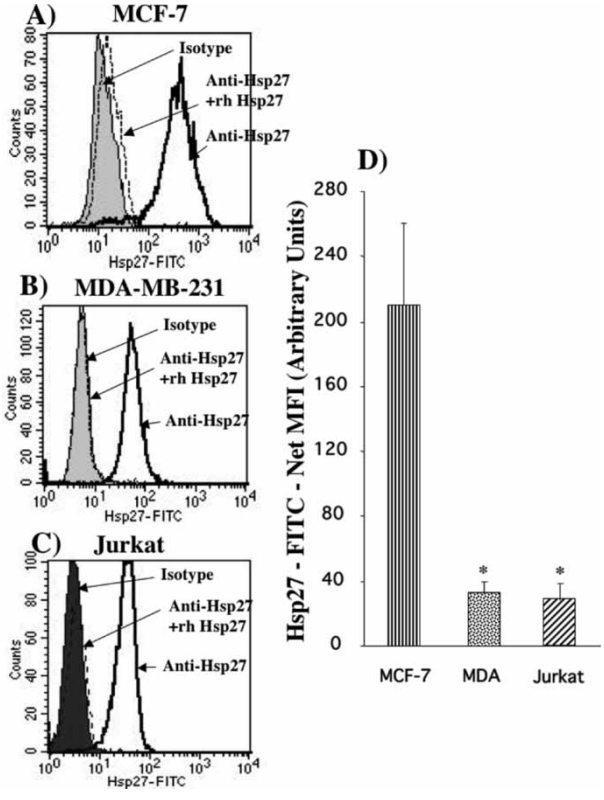

Figure 2. Flow cytometric determination of cellular distribution and specific expression of hsp27 in (A) MCF-7, (B) MDA-MB-231 and (C) Jurkat cells. Intracellular hsp27 was assessed by fixation and permeabilization of the cells followed by staining with FITC labeled anti-hsp27 antibody or matching isotype control. Specificity of antibody was assessed by incubation of the antibody with recombinant hsp27 before the addition of the antibody to the cells. The shaded curves represent isotype control, blank curve under the solid line represent the hsp27 antibody data, and the blank curve under the dotted line tests the specificity of the hsp27 antibody. Individual curves are marked with arrows. D) Net mean fluorescence intensity (MFI) data of intracellular hsp27 (as calculated by subtraction of the isotype control data from the hsp27 antibody data) is calculated from n = 5 samples [*p = 0.008 (as compared to MCF-7 cells)].

of hsp27 than MDA-MB-231 cells, almost equal percentage of both cell types ($93 \pm 3.4\%$ MCF-7 and $97 \pm 1\%$ MDA-MB-231) expressed intracellular hsp27 (Figure 2A & B). However, the net MFI of intracellular hsp27 was still significantly ($p = 0.008$; 6.3 fold) higher in MCF-7 cells than in MDA-MB-231 cells (Figure 2D). Interestingly, although Jurkat cell expression of hsp27 was undetectable by Western blotting, flow cytometric measurement could detect intracellular hsp27 levels in most of the Jurkat cells ($95.5 \pm 1.9\%$) although the net MFI for Jurkat hsp27 levels was significantly ($p = 0.008$) lower than that of MCF-7 (Figure 2D).

Specificity of Flow Cytometric Assay

These flow cytometric data are highly specific since incubation of FITC-conjugated anti-hsp27 antibody with recombinant hsp27 blocked $>97\%$ of the antibody binding to the cells (Figures 2A, 2B, and 2C). The flow cytometric data suggests that reduced intracellular hsp27 expression in MDA-MB-231 and Jurkat cells is not due to a lack of hsp27 expression in a subset of cells, but rather due to reduced, overall expression of hsp27 in the entire cell population.

DISCUSSION

Our study demonstrates that flow cytometric measurement of intracellular hsp27 is a more sensitive method than the conventional method of Western blotting. The use of this method not only allows the assessment of overall levels of intracellular hsp27, but also allows for potential identification of hsp27 expressing (or lacking) subpopulations within the same sample. It also requires 5-fold fewer cells than immunblotting and could be performed much more rapidly than Western blotting (1–2 days vs about 2 hours for flow cytometry).

Our Western blotting data is similar to the previous reports for MBA-MB-231's and MCF-7's hsp27 levels.[11,18–20] However, the quantitative intracellular hsp27 data obtained from the immunoblotting method (19-fold higher band intensity in MCF-7 cells than MBA-MB-231 cells) was different from the flow cytometry based data (6.3-fold higher net MFI in MCF-7 cells than MDA-MB-231 cells). In contrast, flow cytometric results for hsp27 protein are somewhat similar to the mRNA results (6-fold differential expression of hsp27 mRNA in MCF-7 cells than MDA-MB-231 cells) as described by Kuang et al.[20] The possible reason for the discrepancy between the immunoblotting and flow cytometry data is that the flow cytometric measurements for MFI data is much more sensitive than the immunoblotting assay.

In two out of five immunoblotting experiments, the band for hsp27 in MDA-MB-231 (from 25 μg of total protein run on a mini gel) could barely

be detected. In contrast, hsp27 levels could sensitively be detected in MDA-MB-231 cells in all 5 experiments by flow cytometry, indicating the greater sensitivity of flow cytometry for the detection of hsp27 over immunoblotting. Moreover, the Jurkat cells that did not exhibit any hsp27 band in any of the five immunoblots, showed hsp27 positivity in all five flow cytometry experiments, again indicating the sensitivity and greater validity of flow cytometry data over the conventional immunobloting data for quantitative estimation of hsp27. In fact, when the hsp27 levels in a particular cell type are extremely low as compared to another cell type with abundant hsp27 expression, data obtained by immunoblotting becomes more qualitative rather than quantitative.

Recently, some commercial ELISA kits became available which can detect hsp27 levels in cell lysates. We could not detect any hsp27 protein in Jurkat cell lysates using an hsp27 ELISA kit from Oncogene Research Products. In fact, the data sheet of this ELISA kit clearly supports our finding that hsp27 level is not detectable in Jurkat cells by this kit. In addition, in one experiment using the sensitive ELISA method developed in our laboratory for measurement of serum hsp27 levels we could successfully detect hsp27 levels in MCF-7 and MDA-MB-231 lysates but not in Jurkat lysate (data not shown). Thus, flow cytometric method may be more sensitive than ELISA, as well as Western Blotting, for measurement of hsp27 levels. Moreover, the flow cytometric method can detect intracellular hsp27 levels at the individual cellular level, which is not possible by either Western blotting or ELISA. Use of this rapid, as well as sensitive, method for measurement of hsp27 levels in isolated tumor cells can be implemented at the clinic for breast cancer patients. In addition, this sensitive and quantitative method may provide better correlation of the tumor cell hsp27 levels to patients' responses to therapy and overall survival as opposed to conflicting reports of clinical outcome data on the basis of hsp27 measurement in tumor sections by immunohistochemistry or in tumor lysates by Western Blotting.

ABBREVIATIONS

Hsp27, heat shock protein 27; MFI, mean fluorescence intensity; ELISA, enzyme linked immunosorbent assay; FITC, fluorescence isothiocyanate; ECL, enhanced chemiluminescence; BSA, bovine serum albumin; HRP, horseradish peroixidase.

ACKNOWLEDGMENTS

The research was supported by a grant from Susan G. Komen Breast Cancer Foundation. We sincerely acknowledge the technical assistance provided by

Ms. Sarah Roach and Ms. Kristie Stiles. We also acknowledge the sincere co-operation of Dr. Carol Miller-Graziano, the Director of Surgical Research.

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Received January 17, 2007
Accepted February 20, 2007
Manuscript 3227