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Cerebrospinal fluid levels of extracellular heat shock protein 72: A potential biomarker for bacterial meningitis in children

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Abstract. Extracellular heat shock protein 72 (Hsp72) is an endogenous danger signal and potential biomarker for critical illness in children. We hypothesized that elevated levels of extracellular Hsp72 in the cerebrospinal fluid (CSF) of children with suspected meningitis could predict bacterial meningitis. We measured extracellular Hsp72 levels in the CSF of 31 critically ill children with suspected meningitis via a commercially available enzyme-linked immunosorbent assay. Fourteen had bacterial meningitis based on CSF pleocytosis and bacterial growth in either blood or CSF culture. Seventeen children with negative cultures comprised the control group. CSF Hsp72 was significantly elevated in children with bacterial meningitis compared to controls. Importantly, CSF Hsp72 levels did not correlate with the CSF white blood cell count. On receiver operator characteristic analysis, using a cut-off of 8.1 ng/mL, CSF Hsp72 has a sensitivity of 79% and a specificity of 94% for predicting bacterial meningitis. We therefore conclude that CSF extracellular Hsp72 levels are elevated in critically ill children with bacterial meningitis versus controls. Hsp72 potentially offers clinicians improved diagnostic information in distinguishing bacterial meningitis from other processes.

Keywords: Heat shock proteins, danger signals, stress proteins, meningitis, infection, cerebrospinal fluid, biomarker, extracellular fluid

1. Introduction

The heat shock proteins (Hsps) are a group of highly conserved polypeptides whose function is to activate and mediate critical cellular stress response programs. Originally discovered and characterized after exposure of cells to elevated temperatures [1], it is now known that many stressors induce expression of these essential survival proteins, including hypoxia, acidosis, ischemia-reperfusion injury, reactive oxygen species, and infection [2]. The primary intracellular role of the Hsps, as initially described, is to function as

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molecular chaperones, facilitating the correct folding and assembly of nascent polypeptide chains, stabilizing and correcting protein tertiary and quaternary structure in the face of denaturing stressors, and preventing pathologic protein aggregation [3, 4]. Hsps have been found in virtually every cellular compartment and in every tissue, where many are constitutively expressed at some level, indicating the importance of their role in maintaining cellular homeostasis. Levels of the various Hsps increase dramatically in response to cellular injury to accommodate increased utilization and to subserve additional functions. This is now known as the heat shock response, or stress response. Importantly, induction of the stress response appears to confer protection against a wide variety of cell stressors, which has been called stress tolerance [5]. Beyond their role as molecular chaperones, and perhaps more relevant to critical illness is the fact that Hsps have been shown to decrease pro-inflammatory signaling and cytokine production via suppression of the nuclear factor-kappa B transcription factors, specifically by stabilizing nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-α [5–9]. This is likely one of the mechanisms, whereby the heat shock response induces stress tolerance and protects cells from secondary insults [2].

While classically considered an intracellular protein, many studies over the last several years have found that Hsps can also be present in the extracellular compartment. Clinical studies have documented Hsps in a variety of disorders in almost every extracellular fluid examined to date, including serum, plasma [10], urine [11, 12], bronchoalveolar lavage fluid [13], amniotic fluid [14], and cerebrospinal fluid (CSF) [15]. For example, serum levels of the 72 kDa Hsp (Hsp72) are increased in critically ill patients following trauma and severe sepsis [10, 16, 17]. In most of these studies, increased extracellular Hsp72 levels correlate directly with severity of illness and mortality. As such, several investigators have hypothesized that Hsp72 functions as a "danger signal" to alert the immune system of tissue damage or illness [13, 18–21]. Consistent with this assertion, extracellular Hsp72 has been shown to play an active role in initiating and modulating the inflammatory response [8, 13, 22–24]. Taken together, these data indicate that Hsp72 is integrally involved in the immune response to injury and infection and thus is a potential biomarker of critical illness. With that background in mind, we hypothesized that CSF levels of extracellular Hsp72 would be increased in critically ill children with bacterial meningitis and could therefore serve as a diagnostic biomarker.

2. Materials and methods

2.1. Clinical study

Following Institutional Review Board approval and with informed consent, we measured extracellular Hsp72 levels in CSF samples obtained from a previously published, prospective study in which CSF samples were obtained from a convenience sample of critically ill children (aged 0–18 yr) undergoing lumbar puncture for suspected meningitis at the Children's Medical Center of Dallas [25]. We prospectively defined bacterial meningitis as a CSF white blood cell (WBC) count >10 cells/mm^3 in the presence of either a CSF or blood culture positive for bacterial growth. All other children were included in the control group if they did not meet these criteria. Many of the children in the control group had CSF pleocytosis, but negative culture results and were later found to have viral etiologies for their illness.

2.2. Enzyme-linked immunosorbent assay (ELISA)

After standard laboratory studies were performed, each CSF sample was centrifuged and the supernatant collected and frozen at −70°C for later study. CSF samples subsequently underwent batch analysis for extracellular Hsp72 using a commercially available ELISA kit (StressGen/Enzo Life Sciences, Plymouth Meeting, PA).

2.3. Statistical analysis

Data were analyzed using Stata/SE 11.1 (StataCorp, College Station, TX). Non-parametric, continuous data were expressed as median and interquartile ranges (IQR) and were compared using Mann-Whitney *U* test. Spearman correlation coefficient was calculated between CSF WBC count and Hsp72 levels. Receiver operating characteristic (ROC) curves were then constructed for each biomarker. Specificity, sensitivity, positive predictive value, negative predictive value, and likelihood ratios were calculated using standard formulas. A P value <0.05 was considered statistically significant.

3. Results

CSF samples from 31 children with suspected bacterial meningitis who were enrolled in the original study [25] were available for analysis. The clinical characteristics of each group are detailed in Table 1. Ultimately, 14 children were determined to have bacterial meningitis based on CSF pleocytosis and culture results. The most frequently identified causative organisms were *Neisseria meningitidis* and *Streptococcus pneumoniae*. The remaining 17 children, identified as controls, had disparate causes for their symptoms including viral respiratory tract infections, viral/aseptic meningitis, Kawasaki disease, and dehydration. The mean age of both groups was less than 1 yr and each group had a proportional representation of both genders. Neither characteristic was statistically different between the two populations. As expected, the CSF WBC count was significantly elevated in the bacterial meningitis group compared to the control population (748; IQR 50, 4490 vs. 28; IQR 2, 126 respectively; $P = 0.004$).

Extracellular Hsp72 levels were measured in the CSF samples by ELISA. Children in the bacterial meningitis group had a median CSF Hsp72 concentration of 12.95 ng/mL (IQR 8.33, 21.8), compared to a median CSF Hsp72 level of 2.24 ng/mL (IQR 0.97, 3.16) in the control group (Fig. 1, $P = 0.0002$). Previous studies have suggested that one potential source of extracellular Hsp72 is release from macrophages, monocytes, and neutrophils [26, 27]. Importantly, with this in mind, we were unable to show a significant correlation between CSF Hsp72 and CSF WBC count (R^2 0.22, $P =$ Not significant), strongly suggesting that the higher levels of CSF Hsp72 in the bacterial meningitis group are not merely due to the higher CSF WBC count in this group. ROC analysis of CSF Hsp72 levels yielded an area under the curve (AUC) of 0.90 (95% confidence interval [CI] 0.74, 0.98, $P < 0.05$), indicating the potential to perform very well as a biomarker of bacterial meningitis (Fig. 2). In comparison, ROC analysis of the CSF WBC count demonstrated an AUC of 0.80 (95% CI 0.63, 0.93, $P < 0.05$) (Fig. 3). Direct statistical comparison between the two AUCs showed that CSF Hsp72 performed equally well to CSF WBC ($P = 0.13$). ROC analysis further yielded a best "cut-off" for CSF Hsp72 for diagnosing bacterial meningitis of 8.1 ng/mL, and 28 cells/mm^3 for CSF WBC (Table 2).

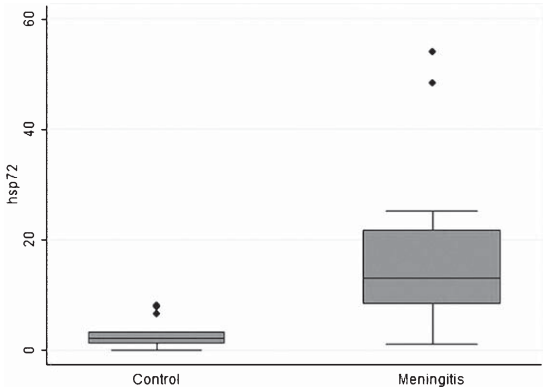

Fig. 1. Cerebrospinal fluid heat shock protein 72 levels are significantly higher in the group of children with bacterial meningitis ($P = 0.0002$).

4. Discussion

In this study, we show that CSF Hsp72 levels are significantly increased in critically ill children with

Table 1
Characteristics of the study groups

Characteristics	Bacterial meningitis	Control
Number of patients	14	17
Age (years)	0.8 (interquartile ranges 0.25, 2.1)	0.3 (interquartile ranges 0.17, 2.4)
Male:female ratio	7:7	10:7
Cerebrospinal fluid white blood cell count (cells/mm^3)	748 (interquartile ranges 50, 4490)	28 (interquartile ranges 2, 126)*
Etiology**	*Neisseria* meningitidis	Viral respiratory tract infection
	Streptococcus pneumoniae	Aseptic meningitis
	Streptococcus bovis	Enteroviral meningitis
	Group A streptococcus	Kawasaki disease
	Group B streptococcus	Hyponatremia/dehydration
	Mycobacterium tuberculosis	

*Only the cerebrospinal fluid white blood cell count was statistically different between the bacterial meningitis and control groups ($P = 0.004$).
**Most commonly identified etiologies of illness.

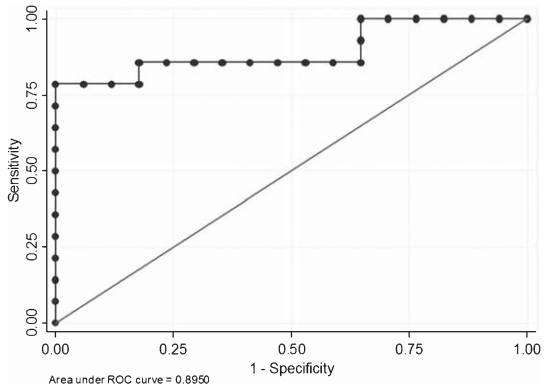

Fig. 2. Receiver operator characteristic analysis for heat shock protein 72 demonstrates a very good area under the curve of 0.90.

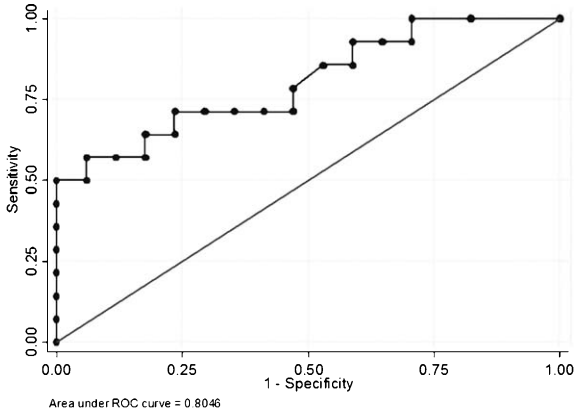

Fig. 3. Receiver operator characteristic analysis for cerebrospinal fluid white blood cell count reveals an area under the curve of 0.80.

bacterial meningitis compared to controls. Importantly, the CSF Hsp72 levels did not correlate with the CSF WBC count, indicating that CSF Hsp72 levels are independent of the WBC count. This differs from the findings of Tang et al. [15] whose data showed some correlation between CSF Hsp72 levels and WBC count. That group, however, relied upon a semiquantitative Western blot assay to determine Hsp72 concentration, whereas we employed a highly sensitive ELISA, which is more likely to yield accurate results. On ROC analysis, CSF Hsp72 levels had a slightly better AUC value (though not a statistically significant difference) than that for the CSF WBC count, suggesting that CSF Hsp72 is a potential biomarker for bacterial meningitis that performs at least as equally well to the "gold standard" of CSF WBC. Notably, other CSF pro-inflammatory cytokines were also significantly increased in the CSF of patients with bacterial meningitis.

These findings are important because employing accessory indicators of bacterial disease may help improve diagnostic accuracy in clinical settings where uncertainty exists with standard CSF tests (protein, glucose, WBC count). Pediatric health care providers often encounter ill appearing children with mild to moderate CSF pleocytosis. Blood and CSF cultures take 1-2 d to result and do not always provide confirmation of infection. This diagnostic scenario becomes more complicated if the child has received pretreatment with antibiotics, which may clear the CSF of bacteria within 1-2 h of administration [28]. Distinguishing bacterial meningitis from nonbacterial causes of pediatric illness has important ramifications for treatment including the selection and duration of antibiotic therapy. Developing a way to assay supplemental biomarkers either at the point of care or within a short time from sample collection could significantly improve the individualization of therapy and prevent unnecessary antibiotic administration.

The primary strength of this study was that it was conducted in a clinical setting immediately relevant to real-life management decision-making. Children with bacterial meningitis were compared not with healthy controls, but rather to children who presented with similar symptoms. The fact that these assays were able to distinguish bacterial meningitis from amongst

Table 2
CSF heat shock protein 72 compared to the CSF WBC count for the diagnosis of bacterial meningitis

Characteristics	CSF Hsp 72	CSF WBC count
Best "cut off"	8.1 ng/mL	28 cells/mm^3
Sensitivity	79% (95% CI 49, 94%)	86% (95% CI 56, 97%)
Specificity	94% (95% CI 69, 100%)	47% (95% CI 24, 71%)
Positive predictive value	92% (95% CI 60, 100%)	57% (95% CI 34, 77%)
Negative predictive value	84% (95% CI 60, 96%)	80% (95% CI 44, 96%)
Positive likelihood ratio	13.4 (95% CI 1.96, 91.2)	1.62 (95% CI 0.99, 2.66)
Negative likelihood ratio	0.23 (95% CI 0.08, 0.63)	0.30 (95% CI 0.07, 1.25)

CSF = Cerebrospinal fluid; WBC = White blood cell; CI = Confidence interval.

a group of children, all of which were ill, indicate their applicability to the day-to-day scenarios most often encountered by pediatric health care providers.

Notable limitations to this investigation include its small sample size and lack of independent validation. An additional problem with these findings rests with their potential application in clinical practice. The assay required to measure CSF Hsp72 is not difficult, but requires specialized reagents and equipment not currently available in most clinical laboratories and definitely not yet at the bedside. This is an important area of future research to bring these findings into the clinical arena. Finally, the population studied was limited to infants less than 2 yr of age. Further studies will be required to examine the role of CSF Hsp72 as a biomarker for older patients with bacterial meningitis.

In summary, we have demonstrated that CSF levels of Hsp72 are increased in children with bacterial meningitis compared with controls. ROC analysis of CSF Hsp72 indicate that it offers clinicians additional diagnostic information in distinguishing patients with bacterial meningitis from those without.

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