RESEARCH ARTICLE

Evidence of Multimeric Forms of HSP70 with Phosphorylation on Serine and Tyrosine Residues – Implications for Roles of HSP70 in Detection of GI Cancers

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Abstract

Background: Heat-shock protein70 (HSP70) are intracellular protein chaperones, with emerging evidence of their association with various diseases. We have previously reported significantly elevated plasma-HSP70 (pHSP70) in pancreatic cancer. Current methods of pHSP70 isolation are ELISA-based which lack specificity due to cross-reactivity by similarities in the amino-acid sequence in regions of the protein backbone resulting in overestimated HSP70 value. Materials and Methods: This study was undertaken to develop a methodology to capture all isoforms of pHSP70, while further defining their tyrosine and serine phosphorylation status. Results: The methodology included gel electrophoresis on centrifuged supernatant obtained from plasma incubated with HSP70 antibody-coupled beads. After blocking non-specific binding sites, blots were immunostained with monoclonal-antibody specific for human-HSP70, phosphoserine and phosphotyrosine. Conclusions: Our novel immunocapture approach has distinct advantages over the commercially available methods of pHSP70 quantification by allowing isolation of molecular aggregates of HSP70 with additional ability to precisely distinguish phosphorylation state of HSP70 molecules at serine and tyrosine residues.

Keywords: HSP70 - multimeric forms - phosphorylation - serine - tyrosine - GI cancer detection

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Introduction

Heat-shock protein70 (HSP70) are intracellular protein chaperones, with emerging evidence of their association with various diseases. We have previously reported significantly elevated plasma-HSP70 (pHSP70) in pancreatic cancer. Current methods of pHSP70 isolation are ELISA-based which lack specificity due to cross-reactivity by similarities in the amino-acid sequence in regions of the protein backbone resulting in overestimated HSP70 value. This study was undertaken to develop a methodology to capture all isoforms of pHSP70, while further defining their tyrosine and serine phosphorylation status. The methodology included gel electrophoresis on centrifuged supernatant obtained from plasma incubated with HSP70 antibody-coupled beads. After blocking non-specific binding sites, blots were immunostained with monoclonal-antibody specific for human-HSP70, phosphoserine and phosphotyrosine. Our novel immunocapture approach has distinct advantages over the commercially available methods of pHSP70 quantification by allowing isolation of molecular aggregates of HSP70 with additional ability to precisely distinguish phosphorylation state of HSP70 molecules at serine and tyrosine residues.

Ductal adenocarcinoma of the pancreas remains a leading cause of cancer deaths in the United States. In an effort to devise an early detection system, we recently reported evidence to show that plasma soluble heat-shock protein 70 (HSP70) is significantly elevated in pancreatic cancer (Dutta et al., 2012). In this study, we demonstrated a novel approach involving the immunocapture of plasma HSP70 and the subsequent demonstration by Western blot of the existence of several isoforms of phosphorylated HSP70 on serine and tyrosine residues. Heat shock proteins (HSPs) are ubiquitous, highly conserved proteins that are found in both prokaryote and eukaryote organisms. These proteins are cytoprotective and are involved in intracellular regulatory pathways, functioning as molecular chaperones for other cell proteins (Rappa et al., 2012). They were first described by Ritossa in 1962, and were thought to be elaborated only in response to thermal stress (Ritossa, 1962). However, this family of proteins is now known to be up-regulated in response to various...
other stimuli including oxygen-derived free radicals, amino-acid analogs, ethanol, heavy metals, ischemia-reperfusion injury and several inflammatory states, thus participating in wide array of functions in humans (Kiang et al., 1998; Rakonczay et al., 2003; Shamaei-Tousi et al., 2007; Söté et al., 2007; Murphey, 2013). HSPs are classified according to their molecular weight, and the most well understood members of this diverse family fall between the 60 and 110 kDa range. These HSPs are essential to normal cellular function and response to stress, and perform diverse chaperoning functions in cells, including protein folding and peptide aggregation (Smith et al., 1998; Sikora et al., 2007; Bottani et al., 2009; Baird et al., 2012). HSP70 is the most temperature sensitive and highly conserved group of HSPs and is generally regarded as the intracellular molecule that is released into the extracellular environment during pathological states (Sikora et al., 2007; Kaul et al., 2011). However, there is increasing evidence that under several circumstances these proteins can be released from cells in the absence of cellular necrosis. Furthermore, evidence also suggests that extracellular HSP70 may have a range of poorly understood immune-regulatory activities (Sharp et al., 2001; Joly et al., 2010). It is now clear that soluble HSP70 is present in human serum and has been independently associated with a number of disease states including colorectal cancer, peripheral artery disease and hepatitis C virus-related hepatocellular carcinoma (Takashima et al., 2003; Kocsis et al., 2010; Krepsa et al., 2010). Current methods for isolation of plasma HSP70 include commercially available ELISA based kits. However, these ELISA methods lack specificity because the anti-HSP70 antibody that is used in the assay captures HSP70, and other closely related molecular forms of HSP70. This cross-reactivity is caused by similarities in the amino acid sequence in regions of the protein backbone (sequence homology). As a result, the values obtained by ELISA is a reflection of the entire HSP70 family of cross-reacting proteins and may be invalid as a tool for distinguishing different pathological conditions.

During the course of our work on HSP70, we recognized that the native secreted form of this family of proteins exhibit a considerable degree of pleiomorphism resulting in multimeric aggregates that are recognized by antibodies raised against HSP70. Other members of this family, with their distinctive molecular weights, are also detected by the cross-reacting antibody that recognizes segments of the protein backbone that bear sequence homology. In addition, HSP70 is known to undergo post-translational modification through phosphorylation of serine and tyrosine residues (Dutta et al., 2000). Therefore, in order to distinguish these different molecular forms, we proposed to use electrophoretic mobility as a tool. This work was initiated to develop a procedure that would allow us to distinguish the multimeric forms of HSP70 and also provide us with an estimate of the specificity of the phosphorylation on serine and tyrosine residues. Reliable identification of these additional discriminants may shed light on the pathophysiology of disease processes.

Materials and Methods

This study was undertaken at Sinai Hospital of Baltimore after approval from the Institutional Review Board (IRB). The aims of the study were: i) To develop an immunocapture technique to isolate HSP70 from human plasma; ii) To characterize the nature of immunoreactive HSP70 and its molecular aggregates; and iii) To assess the degree of phosphorylation on serine and tyrosine residues in each species of immunoreactive HSP70.

Patient selection

Six subjects were enrolled into the study after obtaining consent. 10 ml of blood was drawn into collection tubes from each person in a sterile fashion. The plasma was separated and combined with protease and phosphatase inhibitors. Plasma HSP70 was isolated based on a novel approach previously described (Dutta et al., 2000). The steps of plasma HSP70 extraction are summarized below:

i) HSP isolation and extraction from serum: 250 µl of plasma mixed with 12.5 µl pre-made HSP70 antibody-coupled Protein A sepharose beads was incubated at 4°C overnight. Samples were suspended in 50 µl of laemmli buffer and boiled for 8 minutes (in order to elute protein off the coupled beads), centrifuged and the supernatants stored for western blotting.

ii) Preparation of HSP 70 antibody-coupled protein a sepharose bead: 50µl of HSP70 antibody (Sigma Chemicals USA) was bound to 100µg Protein A sepharose beads (Sigma Chemicals USA) for 2 hours with gentle rocking at room temperature. The beads were washed twice with 10 volumes (1 ml) of 0.2M Sodium Borate (pH=9) by centrifuging at 3000g for 5 minutes and re-suspended in 0.2M sodium borate buffer. Enough dimethylpilimidate was added for a 20mM final concentration. The beads were then mixed for 30 minutes at room temperature on a shaker. The reaction was stopped by washing the beads in 0.2M ethanolamine (pH 8.0) and then incubated for 2 hours at room temperature with gentle mixing. After the final wash, the beads were re-suspended in 1ml PBS 0.1% merciolate for long term storage.

iii) Western blotting: aliquots, 10 µl of protein sample were loaded onto 4-20% gradient polyacrylamide gel (Bio-rad, Hercules, CA) and electrophoresis performed at 75V for 2 hours. After gel electrophoresis, the proteins were blotted onto nitrocellulose membranes using mini-trans-blotcell (Bio-Rad, Hercules, CA) at 40 mA for 18 hours. Non-specific binding sites were blocked by incubating blots with 7% non fat dry milk in phosphate buffered...
The present approach allows us to capture all isoforms of HSP70, as well as an estimate of the molecular forms of HSP70, using a Western blot based on tyrosine and serine residues. This technique opens a new window into the biochemical mechanisms of HSP70 expression in its multimeric incarnations as well as defines the nature of the posttranslational modifications by way of phosphorylation on tyrosine and serine residues.

HSPs account for 2-5% of the total protein content of cells under physiological conditions. In response to stress, there is rapid shutdown of the synthesis of most cellular proteins, with a dramatic increase in the expression of HSPs (Rakonczay et al., 2003; Söti et al., 2007; Bottoni et al., 2009; Baird et al., 2012). During periods of physiologic stress, many cellular proteins become partially, or completely, denatured or malfolded (Sikora et al., 2007; Söti et al., 2007). HSPs bind to these damaged proteins, stabilize and refold them, thereby preventing irreversible aggregation (Smith et al., 1998). HSPs also play an important role in the protection of cells against stress induced apoptosis (Dudeja et al., 2009). By virtue of this protective function, HSPs have been shown to prevent acinar cell injury in acute pancreatitis (Bhagat et al., 2000; 2002). HSPs have been shown to be markedly elevated in various cancers when compared with non-transformed cells and their inhibition can induce apoptotic cell death in cancer cells (Aghdassi et al., 2007; Phillips et al., 2007). Furthermore, there is recent data to suggest that soluble HSP70 detected from human plasma can be used as a prognostic marker in patients with colorectal cancer without distant metastases (Kocsis et al., 2010).

HSPs are sub-classified based on their molecular weight into large HSPs, HSP90, HSP70, HSP60, HSP40 and small HSPs. Apart from differences in their molecular weights, they are known to demonstrate polymorphism, a phenomena well described in HSP70 (Dutta et al., 2007). This accounts for several of HSP70s structural and functional differences compared with other members of the HSP family. There is emerging evidence that HSPs can form large multimeric structures by creating stable complexes with folding intermediates of their protein substrates (Pockley et al., 1998; Rea et al., 2001). In turn, these qualities make the quantification of plasma HSP a potential diagnostic or prognostic tool in clinical disease states.

Phosphorylation of HSP70 at serine and tyrosine
roles in mitochondrial protein targeting, particularly in unfolding the newly synthesized protein in the cytosol, maintaining it in an import-competent state and presenting the protein to mitochondrial contact points (Matouschek et al., 2000; Nollen et al., 2002; Robin et al., 2003). Speculatively, phosphorylation of HSP70 at serine and tyrosine residues may enhance mitochondrial targeting by increased affinity for binding to cytoplasmic HSP70. Although the specific clinical relevance of serine and tyrosine phosphorylation is currently unclear, there is data to suggest that the phosphorylation status of chaperone proteins like HSP70 in human breast cancer tissue may help identify a specific phosphoproteomic signature suggesting a common signaling pathway in tumor progression (Lim et al., 2004). In addition, the possibility of a direct role for tyrosine phosphorylation of heat shock proteins in the prometastatic and antiapoptotic phenotypes has been suggested (Xie et al., 2003; Berger et al., 2005). In the current methodology, not only are we able to distinguish HSPs of different molecular weights, but also define their status of phosphorylation, an important insight into the organismal responses of any aberrant pathophysiological processes.

In summary, we describe a novel method of isolation of HSP70 from plasma. The immunoreactive forms of HSP70 are widely distributed in plasma obtained from healthy human subjects, and appear to exist in heterogeneous forms. We have successfully demonstrated the presence of 5 distinct protein bands of HSP70, indicating multimeric forms of this protein. Increased phosphorylation on serine residues as compared with tyrosine residues appears to be a distinct characteristic of immunoreactive HSP70 in human plasma. Apart from describing a new approach for isolation of plasma HSP70, our observations will facilitate further investigation into the nature of secreted HSP70, particularly their states of aggregation necessary for oligomeric assemblies, which will advance the research of pancreatic cancer biomarkers.

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