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Homology of Heat Shock Protein 70 (HSP70) in Human and Mouse Testis

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The 70kDa family of stress proteins is highly conserved showing structural and functional homology. In mammals several isoforms exist: a constitutive isoform of Hsp70 (Hsp73) is thought to act as chaperones for other cellular proteins under non-stress conditions. By contrast, the stress-inducible isoform of Hsp70 (Hsp72) is generally not expressed in unstressed cells; however, upon exposure to stressful conditions, Hsp72 is highly inducible. During the conditions of stress, both Hsp73 and Hsp72 are thought to bind to damaged and misfolded polypeptides, and facilitate their repair. Extracts from mouse, heat-treated and non-treated testes, as well as human testes were obtained, separated electrophoretically, and the Hsp70 protein expression was studied by Western blot analysis. Homology of the two isoforms was detected by employing an absorption method. Polyclonal anti-Hsp70 antibody (DACO) recognized both isoforms in mouse heat-treated testis while in non-treated only constitutive Hsp73 protein was expressed, suggesting that inducible Hsp72 protein appears to repair the proteins whose conformation is altered by stress. After absorption of the antibody with heat-treated mouse testes extracts no specific reaction was registered by Western blotting of mouse and human extracts providing evidence for homology of the Hsp72 and Hsp73 isoforms in these mammalian species.

Key words: heat stress, heat shock proteins, Hsp70, homology, mouse and human testis.

Introduction

The cellular stress response is an evolutionarily conserved defense mechanism characterized by a transcriptionally controlled induction of the synthesis and accumulation of heat shock or stress proteins (HSPs) following exposure of cells to high temperatures or other environmental challenges [5, 10]. The protective effect is the ability of HSPs to act as molecular chaperones, interacting with denatured proteins and helping them to restore their native structure and their function [1]. The stress proteins of the family of 70kDa (HSP70s) are amongst the most highly induced proteins of the cellular stress response in mammals [10]. Hsp70 family encoded by 11 genes in human cells and by 8 genes in murine cells [9]. One of these genes is known to encode the major cytosolic/nuclear constitutive isoform HSP73, whereas two other genes encode the inducible isoform HSP72 [3]. HSP70 shares the property of binding ATP and polypeptides. In the testis, a number of HSP have been identified and characterized, of which HSP70 family

comprises stress-inducible Hsp70 (HSP72) and constitutively expressed Hsc70 (HSP73) [6]. The two isoforms show extensive sequence homology (over 95%), but their expression and functions in male germ cells are still unknown [11]. HSP72 (HSP70) and HSP73 (heat shock cognate, HSC70) have been located within the cytoplasm and, following stress, in the nucleus. High level of Hsp70 expression not only in heat stressed but in normal germ cells during different stages of spermatogenesis suggests its role both in stress conditions and in testis maturation and function [4]. Under physiological conditions, constitutively expressed HSP73 acts as molecular chaperone that assists proper folding, assembly, and intercellular trafficking of newly synthesized proteins. In response to stressful stimuli, HSP72 is induced and involved in cellular repair and protective mechanism [2]. In the last few years it has been demonstrated that this protective effect is in part the result of inhibition of apoptosis or cell suicide [8]. Hsp72 inhibits apoptosis by blocking release of cytochrome *c* from mitochondria. On the other hand, HSP72 is known to inhibit mitochondrial apoptosis-inducing factor (AIF) release, thus decreasing partly cell injury [7]. Heat shock proteins may also regulate the apoptotic pathway downstream of cytochrome *c* release and prior to caspase activation. Binding of cytochrome *c* to Apaf-1 requires significant structural changes to facilitate binding and cleavage of pro-caspase-9 to generate the active enzyme. These structural changes may be inhibited by some Hsps since maintenance of protein structure is a well-established property of Hsps in their role as chaperones. Despite many reports on the protective effects of HSPs, the possible mechanisms by which they negatively modulate the apoptotic process are poorly investigated [8]. The present study focused on monitoring the homology of expressed Hsp73/72 in mouse and human normal mature testis.

Studies on the role of heat shock proteins during human spermatogenesis are still insufficient due to ethic reasons. In this context experiments on homology between certain stress proteins in human and mouse testis are often used as a model system. Our study on homology between human and mouse stress proteins gives the possibility for extrapolation of the results from mouse spermatogenesis towards human spermatogenesis.

Materials and Methods

Extracts from mouse and human testes were used. Pieces from testes of 60-day-old male conventional mature mice (not-treated and treated-incubated under high temperature), and pieces from normal human testes (surgically derived) were prepared in cold Tris-buffered solution (TBS, pH 7.4), homogenized in presence of Protease inhibitors (Sigma Co., St. Louis, USA), and centrifuged. Protein contents were evaluated with Ultraspec 1000.

Protein fractions in extracts from control or heat-treated sexually mature mouse and normal human testes were separated and the expression of homologous antigens was achieved by means of SDS-PAGE and Western blot analysis. Testes extracts were prepared in 6 × Sample buffer in ratio 3:1. Equal amounts of lysate were resolved by 10% SDS polyacrylamide gel under reducing conditions. Protein fractions were visualized by Coomassie Brilliant blue (Sigma Co., USA) and their molecular weight were determined according to standard molecular markers. This was followed by blotting onto nitrocellulose membranes (Amersham Pharmacia Biotech, UK) for 1 hour. Nonspecific binding sites were blocked with 5% nonfat dry milk in TBS for 1 hour. The blots were incubated with primary antibody – rabbit, polyclonal serum against HSP70 (DAKO) at 1:500 overnight at 4 °C. The expression of homologous antigens was detected with the same antibody prepared in two variants: non-absorbed at 1:500, and absorbed extracts from heat treated mouse testis at 1:1000 overnight. Thereafter, the blot was washed and

incubated with biotin-conjugated anti-rabbit IgG at 1:2000 in blocking buffer followed by washing and incubation in streptavidin-alkaline phosphatase for 1 hour. Immunodetection of proteins and homologous antigens were revealed using alkaline-phosphatase buffer (containing 0.1 M Tris, 0.1 M NaCl, 0.005 M $MgCl_2$, pH 9.5) in which were added nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). The reaction was stopping in dH_2O.

Results and Discussion

The present study was conducted to detect the homology of expression of HSP70 in normal and heat-treated mouse, and normal human mature testes. There are described two isoforms of HSP70 – a constitutive HSP73 and an inducible HSP72. Both isoforms share 95% sequence identity and are present in cytosol and nucleus in resting cells, and become predominantly nuclear during or soon after heat stress. We performed SDS-PAGE electrophoresis and corresponding Western blot to detect the expression of HSP70 in extracts of normal mouse and human mature testes (**Fig. 1**).

We observed that under normal conditions antibody against HSP70 recognized

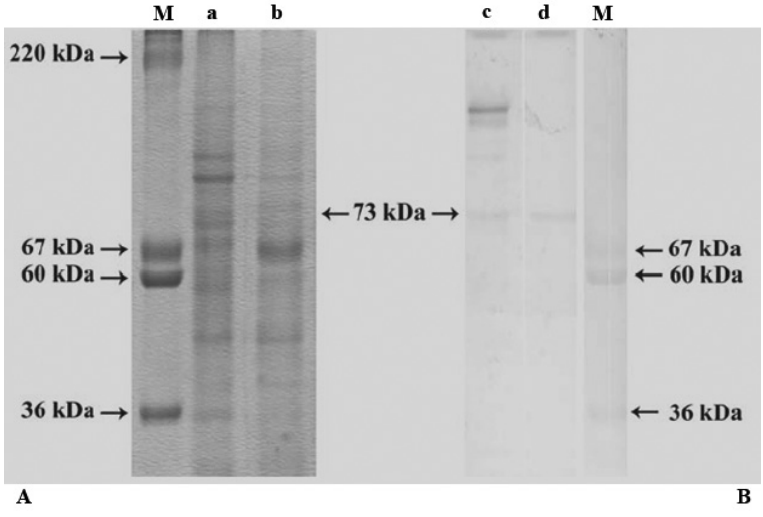

Fig. 1. SDS-PAGE (A) and corresponding Western blot (B) analysis to detect the expression of Hsp70 in extracts of non-treated mouse testis (a, c), and human testis (b, d). Polyclonal anti-Hsp70 antibody recognized protein band corresponding to approximately 73kDa molecular weight in both samples – the constitutive isoform of Hsp70 (Hsp73); (M) – protein molecular weight marker

protein band corresponding to approximately Hsp73 kDa molecular weight – the constitutive isoform of Hsp70 (Hsp73). The results showed that under physiological spermatogenesis, when the process of spontaneous apoptosis exists, Hsp73 is expressed. We assumed that the constitutive isoform Hsp73 has important functions as a molecular chaperone at the time of mouse and human spermatogenesis.

After heat shock the results received from SDS-PAGE and corresponding Western blot of normal and heat-treated mouse testicular extracts revealed protein band respective to Hsp73 isoform. Under hyperthermia conditions the specific reaction of antibody against Hsp73 is increased. We observed enhancement of Hsp73 levels following ex-

posure to heat stress simultaneously with the presence of an additional band, indicates the expression of the inducible Hsp72. We allowed that in male germ cells exists not only the constitutive Hsp70 but also the inducible isoform of this protein. There is evidence that Hsp72 is expressed only under heat inducible apoptosis and probably related with protective functions in spermatogenesis at the time of temperature stress (**Fig.2**).

In our previous study we analyzed the specific expression of the two isoforms

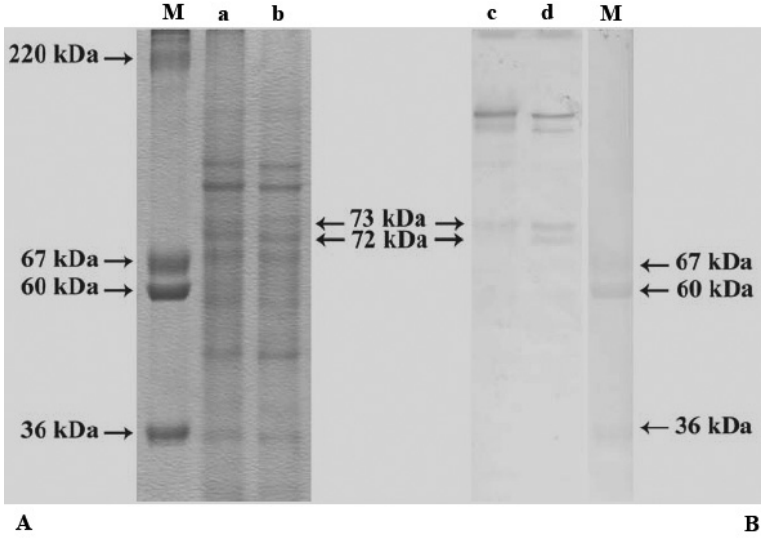

Fig. 2. SDS-PAGE (A) and Western blot (B) of non-treated mouse testis extract (a, c), as well as heat-treated mouse testis extract (b, d). The specific reaction of the antibody against Hsp73 isoform increased after hyperthermia. Note the presence of an additional band in sample from heat-treated tissue, indicates the expression of Hsp72 isoform following the exposure to high temperature. (M) – protein molecular weight marker

(Hsp72/Hsp73) of the Hsp70-gene family during postnatal development of mouse testis and testicular hyperthermia in adult animals, using immunocytochemistry. The obtained results showed expression of Hsp70 in germ cells during all stages of spermatogenesis. Our results confirmed predominant cytoplasmic localization of Hsp70 in spermatogonial cells and in pachytene spermatocytes. Round spermatids expressed Hsp70 not only in the cytoplasm but in the nucleus as well. After heat shock the labeling of the nucleus of spermatocytes and round spermatids was significantly increased, probably due to the movement of Hsp70 from cytoplasm to nucleus. Nuclear translocation of Hsp70 triggers the cascade in signal transduction events leading to realization of chaperone activity of Hsps [4].

We performed SDS-PAGE and respective immunoblot with non-absorbed Ab against Hsp70 in heat-treated mouse, and normal mouse and human testes extracts. The reaction of constitutive Hsp73 isoform was detected in all cases while inducible Hsp72 was expressed only in heat-treated mouse testis (**Fig. 3A**). After absorption of the antibody with heat-treated mouse testes extracts and subsequent Western blot, no specific reaction was registered (**Fig. 3B**). The results obtained with absorption methods together with SDS-PAGE and Western blot analysis are clear evidence for homology

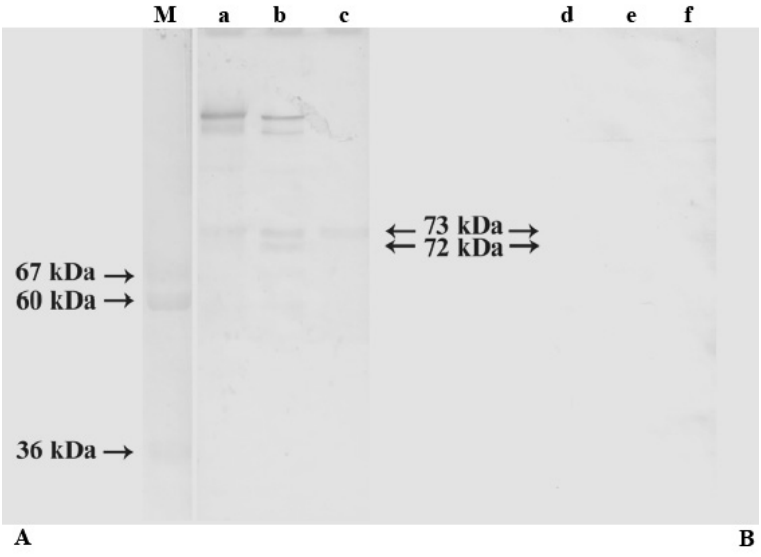

Fig. 3. (A). Western blot analysis of non-treated (a), and heat-treated (b) mouse testes, as well as normal human (c) testes extracts. The reaction of Hsp73 isoform was seen in all samples while the inducible Hsp72 isoform was detected only after heat stress (b); **(B).** Detection of the homology of Hsp70 in mouse and human testes was performed by absorption of the antibody against Hsp70 with heat-treated mouse testis, and subsequent Western blot. After absorption, no specific reaction of the antibody was registered in non-treated (d), heat-treated (e) mouse testes, and normal human testes (f). (M) – protein molecular weight marker

between the two isoforms, Hsp73 and Hsp72, of heat shock protein 70 in mouse and human testes.

Conclusion

In conclusion, the results reported in this paper show that Hsp70 Ab absorbed with heat-treated mouse testis extract in immunoblot does not react with both mouse and human extracts, and provide evidence for homology of Hsp72/73 isoforms in human and mouse testes. Under stress conditions the protein expression of the constitutive isoform Hsp73 increased, simultaneously with the activation of the inducible isoform Hsp72 in order to ensure germ cell survival and to avoid subsequent male infertility problems.

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