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Expression of Hsp27 and Phosphorylated Hsp27 in 8 Weeks Old Human Embryo

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Heat shock proteins (Hsp) interact with many different molecules and play an important role in various cellular functions such as stress tolerance, protein folding, protein degradation, cytoskeletal integrity, signal transduction, cell cycle. The aim of our work is to register the expression of small heat shock protein (sHsp) member's – Hsp27 and phosphorylated Hsp27 (pHsp27), in different tissues and organs of 8-week old human embryo. Immunohistochemical evaluation of human embryo sample was performed using antibodies against Hsp27 and pHsp27, and polymer-based detection system. Results were read using light microscopy.The Hsp27 had cytoplasmic expression in the encephalon, medulla spinalis, skeletal muscles, heart, liver, intestinal epithelium and muscles. The phosphorylated Hsp27 was expressed in bones and muscles. Our results suggest that both forms of Hsp27 play an important specific role in the proliferation and differentiation of different tissue's cells during human embryo development.

Key words: human embryo, Hsp27, pHsp27, small heat shock proteins, immunohistochemistry

Introduction

During the evolution, the living forms created different mechanisms to protect their cells against stress conditions such as environmental, metabolic, pathophysiologic (e.g., heat, cold, oxidative stress, acidosis, ischemia, toxins, heavy metals) changes and abnormalities. The different types of stress forces inflict damage on the macromolecules (proteins denaturation and aggregation) and the cellular stress response is based on repairing the macromolecule structure without regard to the primary source [15]. The most ancient mechanism of cell protection which emerged in the course of evolution is the synthesis of stress proteins (heat shock proteins – Hsps). Hsps are highly conserved proteins, expressed in all organisms, from prokaryotes to man. They interact with many different molecules and play an important role in diverse cellular functions such as stress tolerance, protein folding, protein degradation, maintaining cytoskeletal integrity, cell cycle, autophagy, cell death, differentiation, signal transduction and development [3].

Heat shock proteins are grouped into five main families based on molecular weight, amino acid sequence and function: 100–110 kDa family; the 83–90 kDa family; the 70 kDa family containing Hsps ranging from 66–78 kDa; the 60 kDa family; and the small Hsps ranging from 15–30 kDa.

A prominent group of the stress proteins are the molecular chaperones. The small heat shock proteins are molecular chaperons present in all organisms - from bacteria to men. The most important members of this group are alpha crystallin (HspB5) and Hsp27 (HspB1). Proteins of this family are grouped together based on similar structural and functional properties. They have a conserved core domain, so-called alpha-crystallin-domain (ACD, Hsp20 domain), which is flanked by highly variable sequences that are very important for their chaperone functions, therefore they are the least conserved Hsps despite that they are present in all living organisms [11]. Hsp27 is expressed constitutively in many tissues and cell lines, and its expression increases at high levels after various types of stress. It modulates cell survival during the stress [1].

Bakthisaran et al. [3] suggested that circulating αB crystallin and Hsp27 in the blood plasma may exhibit immunomodulatory and anti-inflammatory functions.

Small Hsps share the ability to form oligomeric structures [2] and are often detected as phosphoproteins.

Phosphorylation of Hsp27 was shown to modulate the functional activity of the protein in a variety of studies. The role of Hsp27 phosphorylation in cell protection is not entirely clear. There are conflicting results in literature concerning this aspect. Some *in vitro* studies concluded that the unphosphorylated oligomeric Hsp27 protects protein against aggregation better, whereas others found no difference or better protection by phosphorylated Hsp27 [12].

Expression of multiple Hsps is required during the mammalian embryo development which is characterized by rapid cell growth and differentiation [27].The mammalian genome is activated during early cleavage, and production of proteins important for cleavage occurs. There are many investigations concerning embryonic expression of Hsp in mammals. Trifonova et al. [25] demonstrated the expression of another small Hsp – alpha crystalline in a variety of organs and tissues of human fetus, which serves as an example of the significance of this protein group in the embryo development.

The expression of small Hsp in significant quantities could be observed in adult as well. Mineva et al. [19] demonstrated pattern of Hsp in both normal and pathological thyroid glands. They proposed that Hsp plays a major role in cell differentiation. This notion is further confirmed by Pupaki et al. [22] which examined the role of Hsp in cell differentiation in pig embryos. On the other hand, Stamenova et al. [24] confirmed the presence of alpha crystalline in human placenta but found no significant differences in the expression or distribution when comparing normal or pathological organs (except a total of two cases).

Hsps are among the first proteins produced in the very early-stage embryos. In the mouse embryo, zygotic genome activation starts at 2-cell stage, and it's prooved that Hsp70.1 is one of first proteins, produced after activation of zygotic genome [4]. Small Hsps are far less studied in mammalian embryos. Due to ethical and technical difficulties, studies about Hsps are focused on early mammalian development.

Hsp27 are registered in murine zygote and 2-, 4-, 8-cells blastomeres [16]. Hsp27 is located in cytoplasm of zygotes and early embryos from the 2-cell stage to blastocyst stage and in the nuclei, but not in nucleoli [16].

Other authors investigated Hsp25 (murine Hsp27) in preimplantation stages and concluded that the highest expression of Hsp25 could be found in the blastocyst stage. In normal conditions, they observed cytoplasmic expression of Hsp25, and in chronic heat shock conditions they found that the investigated protein is located in the nucleus [14].

Data in literature are scarce and mainly relate to Hsp27's developmentally regulated manner of expression. The exact role of Hsp27 in unstressed physiological conditions

remains unclear. There are reports that chaperons could be involved in the keeping balance between differentiation and apoptosis, inhibiting apoptosis by regulating upstream signaling pathways [21, 23]. Some authors suggest that the transient expression of Hsp27 is essential for preventing apoptosis of differentiating embryonic stem cells [18].

Most of the knowledge about the function of sHsps in mammalian embryos is based on studies of animals or somatic cells, induced to differentiate. The precise role of Hsps during the human embryonic development is yet to be elucidated.

The aim of the present study is to describe the expression of a member of the sHsps – Hsp27 and his phosphorylated form in different tissues and organs of 8-week old human embryo.

Materials and Methods

In our experiments we investigated the expression of Hsp27 and pHsp27 in tissues of human 8 weeks old embryo using indirect immunohistochemical method, as described by Hristova et al. [13].

Formalin fixed and paraffin embedded embryo from a patient with elective abortion in the 8th gestation week was immunostained. Gestation age was determined with the patient history for last menstrual period, the ultrasound calculations, and the embryo size and morphology on the sections was checked according to the rules in literature [20]. Tissue sections of 3 μm were prepared and placed on an adhesive slides (VWR Micro Slides, Superfrost Plus, USA).

We used as primary antibodies rabbit polyclonal monospecific anti human anti Hsp27 antibody (Elabsience, code № E-AB-31748) and rabbit polyclonal monospecific anti human anti pHsp27 antibody (Santa Cruz Biotechnology, Inc code № ab1426). In negative control the primary antibody was omitted.

Deparaffinization was performed in xylene and ethyl alcohol following standard procedure.The visualisation method is the two-step polymer based En-Vision™ FLEX mini Kit, High pH (Dako, code №K8024). It includes a dextran "skeleton" to which were attached multiple enzyme molecules, and anti-mouse and anti-rabbit secondary antibodies.

The ready sections were observed by light microscope (Zeiss Axioscope 20) using a semiquantitative 4-level scale, including no expression, weak, moderate or strong expression [6].

All procedures were performed at the Laboratory of Reproductive Immunology, Department of Biology, Medical University of Sofia after institutional ethical board approval and in agreement with the Declaration of Helsinki for Medical Research Involving Human Subjects.

Results

After applied indirect immunohistochemical method we observed strong expression of Hsp27 in myocardium **(Fig.1A)** and skeletal muscles **(Fig.1C)**. Moderate to weak cytoplasmic expression of Hsp27 was observed in the centers of ossification**(Fig.1E)**, skin – epidermis and derma **(Fig. 1G)**, smooth intestinal muscle **(Fig. 1I)**, intestinal epithelium **(Fig. 1K)**. We obtained weak cytoplasmic expression of Hsp27 in the hepatocytes **(Fig. 1M)**, the encephalon (**Fig. 1O**) and medulla spinalis.

We registered strong staining of phosphorylated form of Hsp27 the myocardium **(Fig.1B)** and skeletal muscles **(Fig. 1D)**. Strong to moderate cytoplasmic expression of Hsp27 was observed in the centers of ossification **(Fig.1E).** Moderate to weak expression

we found in intestinal smooth muscles **(Fig. 1J)**. Negative immunohistochemical reaction with phosphorylated form of Hsp27 was registered in skin (epidermis and derma) **(Fig. 1H)**, intestinal epithelium **(Fig. 1L)**, liver cells **(Fig.1N)**, and in the encephalon **(Fig. 1P)**.

Nuclear expression of Hsp27 and phosphorylated Hsp27 was registered in single cells of the ossification centers in bones **(Fig. 1E, F)**.

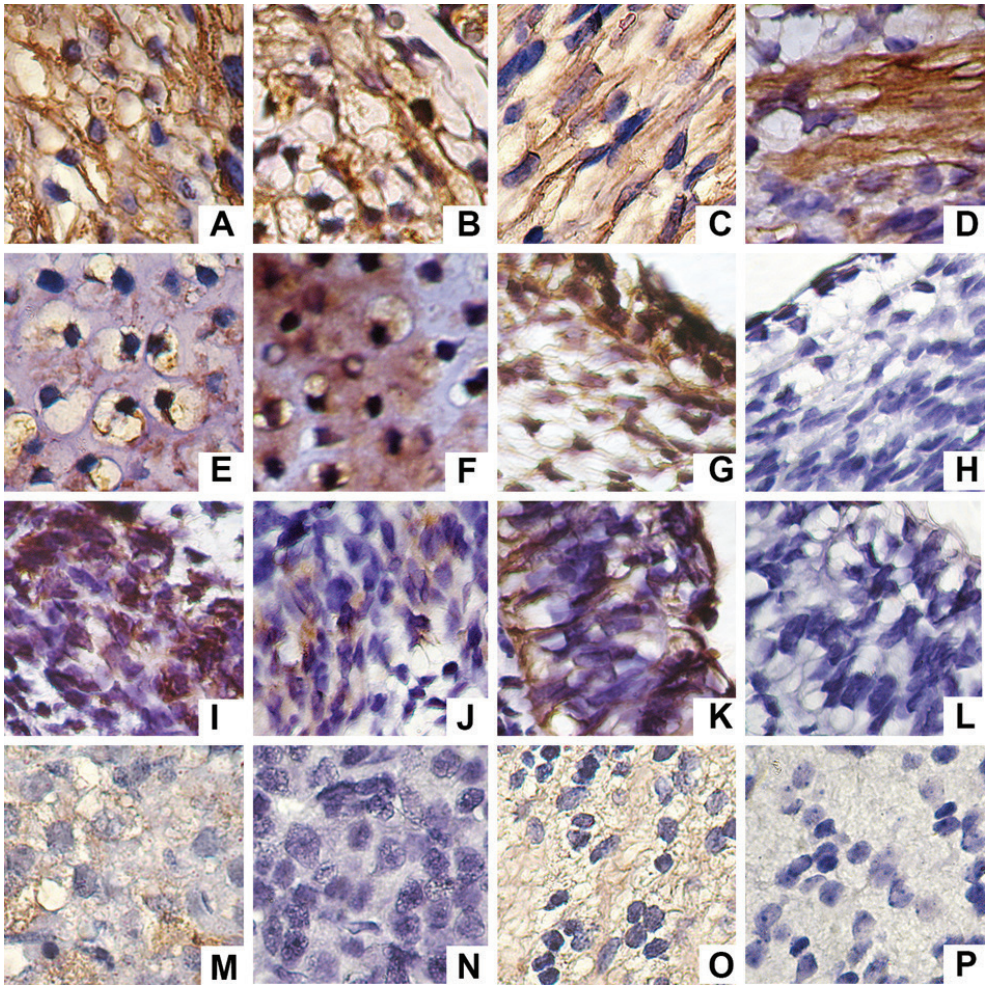

Fig. 1. (A – Hsp27, B – pHsp27) Immunohistochemical study on paraffin embedded tissues of 8 gw human emrbryo. Strong cytoplasmic expression in the myocardium, nuclear expression of the protein is not registered; (C – Hsp27, D – pHsp27) Strong cytoplasmic expression in the skeletal muscles, nuclear expression of the protein is not registered; (E – Hsp27, F – pHsp27) Moderate cytoplasmic and nuclear expression in single cells in ossification centres; (G) Weak cytoplasmic expression of Hsp27 in epidermis and derma of skin; (H) Negative immunohistochemical reaction with pHsp27; (I – Hsp27, J – pHsp27) Moderate to weak cytoplasmic expression in the intestinal smooth muscles, nuclear expression of the protein is not registered.; (K) Moderate to weak cytoplasmic expression of Hsp27 in the intestinal epithelium; (L) Negative immunohistochemical reaction with pHsp27; (M) Weak cytoplasmic expression of Hsp27 in hepatocytes; (N) Negative immunohistochemical reaction with pHsp27; (O) Weak cytoplasmic nevronal expression of Hsp27 in encephalon; (P) Negative immunohistochemical reaction with pHsp27.

Semiquantitative evaluation of immunohistochemical reaction is presented in **Table 1**.

Table 1. Semiquantitative evaluation of cytoplasmic expression of Hsp27 and pHsp27 proteins in organs and tissues of 8 gestation weeks (gw) old human embryo. + weak expression; ++ moderate expression, +++ strong expression; – lack of expression

Tissue	**Hsp27**	**pHsp27**
Skeletal muscles	+++	+++
Heart - myocardium	+++	+++
Bone/ossification center	+	++
Intestine smooth muscle	+	+
Intestinal epithelium	+	–
Encephalon	+	–
Medulla spinalis	+	–
Liver	+	–
Skin – epidermis and derma	+	–
Cartilage	–	–
Lungs	–	–
Kidney	–	–
Thymus	–	–
Nasal epithelium	–	–
Suprarenal gland	–	–
Gonads	–	–

Discussion

The mammalian embryonic development is a very complicated and strongly regulated process, in which Hsps play an important but not fully understood role.

Hsps are proteins, involved in processes of cell proliferation, differentiation and migration [7]. The genes of Hsps cannot be considered as developmental genes but they should be expected to intervene as modulators in the developmental process [7]. The Hsps expression and their functions during development are not yet well described. The central role of Hsps is to act as chaperones. Hsps can bind temporarily different protein molecules and influence their function. Hsp27 limits the activation of apoptosis cascade and reduces the programmed cell death [5]. Garrido C.[10] suggests that the antiapoptotic role of this protein is very important in the processes of proliferation and differentiation during the development of embryo. We admit that this protein has a role not only against stress condition but it plays role in the normal biological processes.

In the present study we investigated sagittal sections of whole 8-week old human embryo and we can observe immunohistochemical evaluation about expression of both forms of Hsp27.

In skeletal and smooth muscles, and myocardium, we registered both forms of Hsp27 – phosphorylated and unphosphorylated. The heart forms early during the embryonic development. It is the first functional organ - beats spontaneously by 4th week. Early expression of some sHSPs (especially orthologues of HSPB1, HSPB8, HSPB5, but also HSPB6 and HSPB2) during heart formation has been reported in a wide range of animal species such as *D. melanogaster*, sea squirt, *D.rerio*, mouse, pig and human. In model object *X. laevis* lack of Hsp27 at the subcellular level causes perturbation of the actin filament network and myofibril disorganization [9]. Some authors [8] assume that Hsp27 is involved in cardiomyocyte differentiation, which is in accordance with our results.

The activity of Hsp27 in skeletal muscles is closely connected with its phosphorylated and unphosphorylated state. Unphosphorylated Hsp27, in its large oligomeric state, is able to bind up to 30 actin monomers, whereas its phosphorylation leads to disintegration of the large complex [9].

An important step of muscle differentiation is fusion of mononucleated myoblasts in multinucleated muscle fibers. Several studies have shown that small HSPs are expressed during the key steps of muscle differentiation. Hsp27 and HspB5 (alpha-B crystallin) make a myotube-specific association with actin microfilaments, which confirms their cytoprotective role. Hsp27 is involved in protection of skeletal myoblasts against oxidative stress and may play an important role in regulating the glutathione system and resistance to Reactive oxygen species (ROS) in skeletal muscle cells [9].

We suggest that strong expression of both states of Hsp27 in skeletal muscles is related with fast growing and differentiation of muscle fibers and his moving activity in this stage.

Small Hsps are very important for connective tissue differentiation during embryonic development. Investigations of chondrogenesis in murine embryos (*in vivo* and *in vitro*) prove intensive expression of Hsp25 during transdifferentiation of chondrocytes in osteoblast-like cells [7]. Our results confirm this observation.

In other organs (liver, brain) we observed only Hsp27, but not phosphorylated form. In the cartilage we detected only phosphorylated form. In the lungs we obtained negative result for both forms of the protein.

Expression of Hsp25 (murine analog of Hsp27 in human) is registered in different stages in mouse embryonic development in brain, in the peripheral tracts and in longitudinal tracts of the ventral region of tegmentum, pons and medulla. Hsp25 is very selectively expressed in isolated or grouped neurons and it is most abundant in axons and dendrites [17]. We observed similar expression of Hsp27 in axons and dendrites.

We compared our results about 8-week embryo with data about expression of Hsp27 in tissues of adults [26] and we continued our previous experiments concerning expression of Hsp27 and pHsp27 in different tissues from human embryos in gestational age from 6 to 12 gestational weeks (gw). We detected the peak of cytoplasmic expression of Hsp27 in embryos in 10 and 11 gw in multiple organs and tissues, that gradually decreased in 12 gw [13]. The phosphorylated form of pHsp27 was weakly expressed from 11gw in single tissues – intestinal muscular layer, lung basal membrane, retinal basal layer, cartilage and nerves [13]. In our previous study we have scarce tissues from 8 gw embryo and information about Hsp27 expression in this age is insufficient.

We observed some differencein Hsp27 expression – in adults Hsp27 lacks in brain cells, according to the data in the literature [26]. In the skin we found a weak to moderate expression of Hsp27, but in adult strong expression is found.

Other difference is observed in gonads – in adults [26] there is a weak expression of Hsp27 in Leydig cells, moderate to strong expression in epididymis glandular cells. In ovaries was observed moderate to strong expression of Hsp27 in follicle cells. We suppose that this result is due to the fact that gonads in 8-week embryo are not yet differentiated as ovary or testis and are not yet functioning.

Conclusions

The present results of the expression of Hsp27 in the tissues and organs of 8 week old human embryo, compared to the results from our previous investigation of tissues and organs from embryos, fetus and adult human give us reason to suppose stage specific expression of Hsp27. This may be due to different needments of the cells during the various stages of their development and function. We suggest that our result will be useful for arranging the complex expression pattern of this protein during embryonic development.

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