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Heat Shock Protein Expression During Gametogenesis and Embryogenesis

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ABSTRACT

When cells are subjected to various stress factors, they increase the production of a group of proteins called heat shock proteins (hsp). Heat shock proteins are highly conserved proteins present in organisms ranging from bacteria to man. Heat shock proteins enable cells to survive adverse environmental conditions by preventing protein denaturation. Thus the physiological and pathological potential of hsps is enormous and has been studied widely over the past two decades. The presence or absence of hsps influences almost every aspect of reproduction. They are among the first proteins produced during mammalian embryo development. In this report, the production of hsps in gametogenesis and early embryo development is described. It has been suggested that prolonged and asymptomatic infections trigger immunity to microbial hsp epitopes that are also expressed in man. This may be relevant for human reproduction, since many couples with fertility problems have had a previous genital tract infection. Antibodies to bacterial and human hsps are present at high titers in sera of many patients undergoing in vitro fertilization. In a mouse embryo culture model, these antibodies impaired the mouse embryo development at unique developmental stages. The gross morphology of these embryos resembled cells undergoing apoptosis. The TUNEL (terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling) staining pattern, which is a common marker of apoptosis, revealed that embryos cultured in the presence of hsp antibodies stained TUNEL-positive more often than unexposed embryos. These data extend pre-existing findings showing the detrimental effect of immune sensitization to hsps on embryo development. Infect. Dis. Obstet. Gynecol. 7:10–16, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS

heat shock proteins; embryo development; in vitro fertilization

Indicators of a cellular heat shock response were first discovered more than 37 years ago, when, in 1962, Ritossa and coworkers described the phenomenon of puffing in salivary gland chromosomes of the fruit fly *Drosophila melanogaster* after exposure to heat.[1] Not many people took notice of this observation, and it was not until 12 years later that the first gene products of this morphological puffing pattern were identified and the term "heat shock proteins" (hsps) created.[2] Today the genes coding for these proteins have been sequenced, their structure described, their chromosomal loca-

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tion defined, and their mode of interaction with nuclear heat shock transcription factors characterized.[3] Studies involving the role of hsp in basic and applied clinical medicine are numerous and involve almost every medical field, including oncology, immunology, and infectious diseases. Drugs modulating the total hsp expression (thus protecting integrity and homeostasis of cells and tissues) are currently in clinical trials.[4,5]

COMMON PROPERTIES OF HEAT SHOCK PROTEINS

A number of crucial characteristics define this group of proteins:

1. All organisms studied ranging from prokaryotic bacteria to mammals, including man, respond to an increase in temperature by switching off the synthesis of most proteins and commencing large-scale synthesis of a few hsps. Even thermophilic organisms, whose optimal growth temperature lies between 50°C and 90°C, respond to sudden temperature increase with the overexpression of hsp.
2. This type of cellular response has not changed very much during evolution. The induced hsps are very similar to one another in very different organisms (their structure has been conserved), and they share a high level of amino-acid sequence homology.
3. The high conservation between very diverse species has important implications for autoimmune diseases.[6]
4. Hsps serve two major functions. First, under physiological conditions, they function as molecular chaperones (intracellular housekeeping proteins) that are involved in mediating the folding of other intracellular proteins (and in some cases their assembly into oligomeric structures). In addition, they have crucial roles in the prevention of inappropriate protein associations and premature folding, intracellular transport, maintenance of proteins in an inactive form, and protein degradation. Second, they are synthesized in response to a wide variety of cellular injuries that are induced by changes in temperature as well as other conditions, such as the presence of free oxygen radicals, infections, heavy metals, ethanol, and ischemia.[7,8]
5. The stress-elicited activation of heat shock genes is called the heat shock response and represents a highly conserved cellular rescue mechanism, which confers additional thermoresistance on stressed cells. Some members of the hsp families are strictly inducible by stress, whereas others are expressed at normal temperature and are only slightly induced by heat shock. The term "hsp" refers to inducible protein products, while "hsc" indicates constitutively (highly homologous with hsp but not heat inducible) expressed chaperones.
6. Heat shock proteins are classified into different families by their molecular weight (kilodalton, kD) rather than by their function. Most scientific knowledge has been accumulated on four families of hsps. These are the "small" hsps: 27kD, 60kD, 70kD, and 90kD. Recently the expression of higher molecular mass hsps during embryo development has also been investigated.[9]
7. Microbial hsps represent dominant antigens. Thus, they are highly immunogenic in humans. Many women have been sensitized during the course of a microbial infection. Antibodies to hsps are especially prevalent in women undergoing infertility treatment.

HEAT SHOCK PROTEINS AND GAMETOGENESIS

Successful spermatogenesis and oogenesis are the two fundamental steps preceding embryo development. Unique expression patterns and functions for hsps have evolved during these processes.

Hsp Expression and Spermatogenesis

During spermatogenesis, three distinct phases can be differentiated: mitotic proliferation of spermatogonia, meiotic development of spermatocytes, and postmeiotic development of spermatids and maturation of the spermatozoon.[10] Since all these developmental stages represent situations where dramatic transformations and cellular differentiation take place, it is not astonishing that spermatogenesis is accompanied by expression of different hsps.[11] During mouse and rat spermatogenesis, the constitutive form of hsp70 (hsc70) accumulates.[12,13] Also the messenger RNA coding for proteins related to hsp86 was found in rat and human testis.[14] In infertile men, it has been demonstrated that the number of hsp60-expressing spermatogo-

nia paralleled the loss of spermatogenic function.[15] These observations suggest that a low level of hsp60 expression in spermatogonia might lead to an altered level of protection, which in turn could be involved in low spermatogenic efficiency. In a recent study, Dix et al. showed for mice that the disruption of the hsp70-2 gene by gene targeting resulted in failed meiosis, germ cell apoptosis, and male infertility.[16] Spermatocytes of mice where the hsp70-2 gene had been knocked out became arrested during meiosis. Morphological examination revealed that these animals had testes only one third the size of control mice. This failure of meiosis was associated with an increase in spermatocyte apoptosis.[17] Thus, hsp70-2 participation during spermatogenesis is required for successful completion of meiosis in mouse spermatocytes.

Hsp Expression During Oogenesis

The female germ line, like the mammalian male germ line, also is sensitive to hyperthermic and other environmental stress factors. Similarly to spermatogenesis, hsp expression appears to play an integral role during oogenesis in a number of species, including insects,[18] fish and amphibians,[19–21] and also mammals.[22] The conservation of hsp expression in evolutionary diverse organisms supports the assumption of the fundamental role of hsp during germ cell development. Hsps are found, for example, in ovarian nurse cells of *Drosophila* where they are subsequently transported to the oocyte.[23] In mammalian oocytes a "window" for heat induction of hsp exists, which is regulated by the specific stage of oocyte development. In mouse oocytes, the heat shock response is maximized during the growth period of the oocyte and declines with the acquisition of the full oocyte size. Finally, it is shut off with terminal oocyte and follicle differentiation.[24,25] Thus, the ability of mouse oocytes to mount an inducible heat shock response is highest during early follicular growth and disappears prior to ovulation. In addition, growing oocytes spontaneously express high levels of the hsc70 and hsp90. Hsc70 is found at high levels in the preovulatory oocyte.[25] Later, its synthesis ceases shortly after germinal vesicle breakdown, and it is undetectable in the ovulated oocyte at the time of fertilization. After meiosis, hsc70 synthesis vanishes completely. This is interesting to note, because it is known that mammalian oocytes are very heat sensitive. Since fully developed oocytes are unable to express the heat inducible hsp70, this could explain why mammalian oocytes exhibit an atypical and degenerate morphology after exposure to hyperthermic stress.[26] The abnormalities included multinuclear eggs and an increase in size of the first polar body. In vitro, elevated temperature reduced the number of oocytes proceeding to metaphase II and decreased the rate of fertilization.[27] The above-described block of heat shock gene induction during oocyte differentiation seems to represent a general feature in oogenesis, even though it may follow different time schedules in different species. It is interesting to speculate about the role of hsp during the ovulation process. Since ovulation is characterized by the cardinal features of an inflammatory reaction,[28] it is possible that hsps play a role in the ovulation process and the maintenance of the postovulatory metabolic activity and survival of the oocyte. However, no data are available for this period of reproduction.

HEAT SHOCK PROTEINS AND EMBRYO DEVELOPMENT

The successful completion of the fertilization process and the initiation of the first cleavage steps mark the beginning of embryo development. Before questioning the role of hsp during embryo development, it is important to realize that almost all of the present knowledge on the function and role of hsp in reproduction relies on information obtained from animal studies or from human cell lines that can be induced to differentiate. Although data from animal models may suggest similar mechanisms in humans, the exact role of hsp for human embryo development remains speculative. Due to technical difficulties and ethical restrictions, most of the existing studies focus on early mammalian embryo development from a zygote up to the expanded trophoblast stage. Less experimental knowledge exists on the role of hsps for advanced embryo and organ development. Since the mouse is often used as a model in the study of mammalian development and since our own data are derived from a mouse embryo model, we will mainly focus on findings concerning the role of hsp in mouse embryo development. Although the hsp70 family has received special attention in this context, members of the 60kD and 90kD hsp families are also

synthesized in the preimplantation embryo of the mouse.[29]

As for all mammals, the embryological development of the mouse can be divided into two main phases. The preimplantation period, which can be easily assessed in vitro, and the postimplantation period. No information is presently available on the role of hsp during early implantation and the attachment to endometrial surfaces. The preimplantation period comprises the time span after ovulation and fertilization in the oviduct before complete implantation in the maternal uterus. In the mouse there is a 4–5-day preimplantation period. During this time embryos develop from one cell (zygote) to the blastocyst (about 100 cells) and migrate freely from the oviduct to the uterus.

Distinctive features of hsp expression are directly linked to major events occurring during the preimplantation phase:

1. Spontaneous hsc70 expression begins with the onset of zygotic genome activity and at the early two-cell stage. During the same period, inducible hsp70 expression is still absent.[29,30]
2. Hsc70 is the predominant hsp70 expressed up to the blastocyst stage.[30]
3. In mouse embryos, the induction of synthesis of hsps by heat shock occurs from the blastocyst stage forward.[31]
4. Since blastocyst formation marks the differentiation of two types of embryonic cells forming the inner cell mass and the outer cell mass, progressive acquisition of inducibility is associated with continuing embryonic differentiation. Thus, inducible hsp70 expression and the formation of the hsp expression appear to be developmentally regulated.

It seems to be a common feature of mammalian embryos that very early stages of development are characterized by a lack of induced hsp synthesis. This inability generally reflects the absence of embryonic gene transcription. As soon as transcription resumes, most heat shock genes become stress inducible. Experiments using nuclei transfers have demonstrated that aging of the egg cytoplasm directs the onset of heat shock gene transcription.[32,33] At the eight-cell stage, the mouse embryo does not yet synthesize inducible hsp70 even after heat shock, but it does synthesize very high levels of the cognate hsc70. However, when an eight-cell-stage nucleus is transferred into a one-cell embryo cytoplast (devoid of its pronuclei), the reconstructed one-cell embryo does not synthesize any hsc70 in the first hours that follow the manipulation. But, after allowing a time for cell division, the reconstructed embryos synthesize both the inducible hsp70 and the cognate hsc70 at the right time relative to the development of the recipient cytoplast. Thus, distinct features of hsp expression are directly linked to major events occurring during the preimplantation phase in mammals.

HUMAN IN VITRO FERTILIZATION, HSPS, AND EMBRYO DEVELOPMENT

Over the past 20 years, progress in assisted reproductive medicine has made in vitro fertilization (IVF) a successful therapy for couples with different causes of infertility. During the IVF procedure, oocytes are recruited, retrieved from the ovary, fertilized, and embryos cultured in vitro. The in vitro conditions for human embryo culture are very similar to the above mentioned experimental in vitro study conditions in animal models.

Although direct evidence is still lacking, one can assume that hsp expression in human embryos is enhanced due to the stressful environmental conditions of in vitro culture. Such embryos have to cope with handling, oxidative stress, variation of temperature, and a completely unique cellular environment. Premature transfer to the uterus at the four- to eight-cell stage may lead to both nutritional and environmental stress. Consequently, in the mouse, hsp70 expression is found to be five- to 15-fold higher in cultured embryos.[34,35] Induced expression of hsp due to environmental factors and constitutive hsp expression may both represent an essential requirement for successful embryo growth in an adverse environment. Overexpression of hsp in this situation is probably to the benefit of the developing embryo. However, failed hsp induction and immunity to hsps could results in detrimental consequences for the growing embryo.

Several previous studies provided evidence that immune sensitization to hsp is associated with unsuccessful embryo development and implantation failure in IVF patients.[36,37] Women undergoing IVF treatment with preexisting local cervical immunity to the hsp60 of *Chlamydia trachomatis* (the most common pathogen responsible for tubal oc-

clusion in these patients) had an increased prevalence of unsuccessful pregnancy outcome.[36] In addition, it could be demonstrated that systemic immunity to human hsp60 was associated with first-trimester spontaneous abortion.[37] In many IVF culture systems, the in vitro fertilized embryos are cultured in medium containing maternal serum. Preexisting hsp antibodies in these sera at high titers could thus compromise the growth potential of developing embryos. In a recent study, antibodies to the most common mammalian hsps exerted a detrimental effect on mouse embryos at unique developmental stages.[38] In the presence of hsp antibodies, embryos became growth arrested and degenerated. Gross morphology of these embryos revealed irregular-sized blastomeres and multiple fragments.

Evaluation of human embryos based on morphological criteria is a common procedure in clinical embryology. The quality of human embryos produced by IVF is variable. Half of the embryos cleave regularly and give equal-sized blastomeres without fragmentation. The remaining embryos often contain variable-sized blastomeres with multiple cellular fragments enclosed within the zonae pellucidae. The degree of fragmentation varies from 5–10% to 100%, and the fragments may be either localized or scattered. Subsequent in vitro development of these fragmented embryos is impaired, often leading to cleavage arrest and embryo degeneration. Upon transfer, these embryos have limited developmental potential and rarely result in pregnancy.[39] Alikani and Cohen[40] looked at the pattern of cell fragmentation in the human embryo as a means of determining the relationship between cell fragmentation and implantation potential. The lowest implantation rate was observed in embryos with large fragments. Viewed through the scanning electron microscope, the surface of these fragments was made up of irregular blebs and protrusions.

Interestingly, programmed cell death (apoptosis) in somatic cells also is first identified as surface blebbing. Blastomeres of fragmented human embryos thus resemble apoptotic cells. Apoptosis is detrimental to blastocyst formation and leads to preimplantation embryo death.[41] Morphologically, apoptosis is characterized by cell shrinkage, chromatin condensation and membrane blebbing. In the final stages, the apoptotic cell becomes fragmented into apoptotic bodies, which are rapidly eliminated by phagocytes. In the early stages of apoptosis, extensive DNA degradation occurs. Cleavage of the DNA may yield double-stranded, low-molecular-weight fragments (mono- and oligonucleosomes), as well as single-strand breaks ("nicks") in the high-molecular-weight DNA. Those DNA strand breaks can be detected by enzymatic labeling with modified nucleotides (dUTP). Terminal deoxynucleotidyl transferase (TdT) labels blunt ends of double-stranded DNA breaks. The end labeling method has also been termed TUNEL (TdT-mediated X-dUTP nick end labeling). The use of fluorescein-dUTP to label the DNA strand breaks allows the detection of the incorporated nucleotides directly with a fluorescence microscope.

MOUSE IN VITRO CO-CULTURE STUDIES

To further assess the impact of hsp antibodies on embryo development, we TUNEL stained murine embryos cultured in the presence of varying concentrations of monoclonal antibodies (10, 50, and 100 ng/mL) to hsp60. To more accurately mimic in vivo conditions of embryonic development, an endometrial co-culture (ECC) system to simulate embryonic development within the uterus was applied. A total of 160 two-cell murine embryos from B6D2F1 mice were grown under two sets of conditions. Half of the embryos were grown utilizing 10% fetal calf serum (FCS) in RPMI in varying concentrations of antibodies to mammalian hsp60. The rest were grown in an ECC system in addition to the same media and antibody concentrations. The ECC tissue was obtained from one fertile patient and consisted of an equal mixture of stromal and glandular cells. Embryonic development to the blastocyst stage (B), hatching stage (H), and outgrowth stage (O) was analyzed and compared between the groups using the Fisher exact test. An additional group of embryos were then grown in 10% FCS in RPMI in either 0 or 100-ng/mL concentrations of antibodies to hsp60 and assessed for the presence of apoptosis as a possible mechanism of toxicity utilizing the TUNEL method.

The control embryos (not grown in antibodies to hsp60 or on ECC) progressed to the B, H, and O stages 95%, 70%, and 70% of the time, respectively. In the study group containing anti-hsp60 antibodies without ECC, embryo growth was in-

hibited at a concentration of 100 ng/mL for each stage of development (B, 25%; H, 15%; and O, 15% of controls, $P < 0.001$). Utilizing the ECC system, embryos not grown in antibodies to hsp60 progressed to the B, H, and O stages 100% of the time, respectively. In this ECC model, toxicity was also not exhibited until a concentration of 100 ng/mL of hsp60 antibody was achieved (B, 80%, P = not significant; H, 70%, $P = 0.02$; and O, 60%, $P = 0.003$ of controls). At the highest concentration of antibodies utilized, the growth inhibition exhibited was always significantly less for the ECC model. Embryos exposed to antibodies to hsp60 stained TUNEL-positive more often than unexposed embryos (30/43 vs. 6/17, $P = 0.03$).

SUMMARY

In a mouse model, we have shown that anti-hsp60 and anti-hsp70 antibodies exhibit a growth inhibiting effect at unique developmental stages of these embryos.[38] A prolonged and undetected infection with bacteria like *C. trachomatis* could induce the production of antibodies to hsps. Women with tubal occlusion undergoing IVF treatment harbor bacterial and human hsp antibodies in their sera at a high rate.[42] The endometrium appears to help modify the toxicity of these antibodies. As demonstrated by TUNEL staining, a possible mechanism of this toxicity may include the induction of apoptosis. Hsps are, as outlined above, essential for successful completion of the single developmental stages of an embryo. The specific expression pattern of hsp may thus play both an essential role for differentiation and a protective role against apoptosis.[9,43] Since embryos exposed to anti-hsp60 stained TUNEL-positive more often than unexposed embryos, it is possible that hsp antibodies may render an embryo more susceptible to apoptosis. However, the precise mechanism of anti-hsp-related inhibition of mouse embryo development and apoptosis is not yet clearly understood, and further studies are needed to clarify the function and clinical relevance of these findings.

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