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Heat shock protein expression and immunity: relevance to gynecologic oncology

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Summary

Heat shock proteins function as molecular chaperones, guiding the transport, assembly and degradation of intracellular polypeptides. Under the influence of non-physiological conditions heat shock protein synthesis is accelerated to aid cell survival. Thus, over-production of heat shock proteins protects malignantly transformed cells from apoptotic cell death and fosters resistance to chemotherapeutic agents and irradiation. Individual heat shock proteins, such as the 27 kDa (hsp27) and 70 kDa (hsp70) heat shock proteins, their antibodies and/or genotypes of polymorphic genes may have diagnostic and prognostic value for different gynecologic malignancies. The possible exploitation of the properties of heat shock proteins for development of unique anti-cancer therapies in individuals resistant to traditional treatment is currently under active investigation.

Key words: Heat shock proteins; Gynecologic cancers; Gene polymorphism; Immune response.

Introduction

The presence of a mechanism that enables cells to respond to environmental stresses in such a way so as to maximize their chances for survival has received a great deal of recent attention. This "stress response" is more commonly known as the "heat shock response" since it was first investigated in cells exposed to elevated temperatures. The proteins induced during a stress response are known as heat shock proteins. Many malignancies can be characterized by the presence of elevated concentrations of heat shock proteins and their presence has been postulated to be central to tumor progression and survival, especially in response to anti-tumor therapies. In addition, several studies have suggested that detection of a specific class of heat shock proteins or their antibodies has prognostic and/or diagnostic value for a particular malignancy. This article will review the classes of heat shock proteins and their biological properties, with an emphasis on mechanisms that might be relevant to tumor biology and immunology. Studies dealing with heat shock proteins in gynecologic malignancies will be highlighted. Lastly, the potential for manipulation of heat shock protein production or immunogenicity for anti-tumor therapy will be discussed.

Heat shock protein families

Heat shock proteins are typically identified according to their molecular weight. A classification of the mammalian heat shock protein families is given in Table 1. Under physiological (non-stressed) conditions, the various heat shock proteins mainly function as molecular chaperones. They transport nascent polypeptides and proteins to various intracellular locations and maintain these molecules in the proper conformation to assure correct protein assembly and folding. In addition, incorrectly folded or denatured proteins are marked by heat shock proteins for degradation and elimination from the cell [1].

It can readily be appreciated that protein assembly and transport may be severely affected when a cell encounters non-physiological conditions. To preserve cell viability under these circumstances, the synthesis of some of the heat shock proteins is up-regulated and their concentration within the cell greatly increases to maximize their protective function. The stress response is initiated by activation of heat shock factor 1

Table 1. — *Classification of mammalian heat shock protein families.*

Name	Location	Constitutive	Inducible	Function
Ubiquitin	cytoplasm, nucleus	+	+	protein degradation
Hsp27	cytoplasm, nucleus	+	+	stabilization of microfilaments, stress tolerance, prevents apoptosis, induced by estrogen
Hsp60	mitochondria	+	+	protein transport and folding
Hsp70	cytoplasm, nucleus	low level	++	protein transport and folding, stress tolerance, prevents apoptosis, binds mutated p53
Hsc70	cytoplasm, nucleus	+	–	protein transport and folding
Gp78	endoplasmic reticulum	+	–	protein transport and folding
Hsp90	cytoplasm, nucleus	+	+	steroid hormone receptor binding, protein folding, signal transduction, regulation of stress response
Gp96	endoplasmic reticulum	+	–	protein transport and signaling
Hsp110	cytoplasm, nucleus	+	+	prevents protein aggregation, stress tolerance

(HSF1). HSF1 exists in an inactive form in the cytoplasm associated with the 90kDa heat shock protein (hsp90). Under conditions of stress, nascent proteins within the cell begin to fold incorrectly. This results in the dissociation of hsp90 from HSF1 and hsp90 binding to the abnormal proteins. As a result of this uncoupling, HSF1 is phosphorylated, undergoes polymerization and is transported to the nucleus. In the nucleus, HSF1 binds to highly conserved nucleotide sequences called heat shock elements (HSE) present in the promoter region of all the inducible heat shock protein genes. This leads to a drastic up-regulation of heat shock protein messenger RNA production and protein synthesis [2].

Heat shock proteins and cell death

There are two general mechanisms leading to the death of cells, necrosis and apoptosis. In necrotic cell death, the cell is exposed to an unmanageable level of stress, regulation of fluid intake is disturbed and the cell swells and bursts. Intracellular contents are released and become accessible to the immune system, leading to a pro-inflammatory systemic and/or localized immune response. In contrast, apoptosis is a genetically programmed mechanism of cell death. In response to specific signals a death program is activated. The affected cell shrinks in volume, the cytoplasmic membrane blebs and the chromatin condenses and becomes fragmented. The cell is engulfed by phagocytes, there is no release of intracellular contents and immune system activation does not occur.

Apoptosis is triggered by two different but overlapping mechanisms, each of which is inhibited by a different heat shock protein. There are receptors on the cell surface, such as the Fas receptor, which upon binding its specific ligand or anti-receptor antibody activates the death cascade. Hsp27 specifically inhibits apoptosis that is initiated by activation of cell surface death receptors [2]. The second mechanism of apoptosis is in response to intracellular oxidative stress. The increased production of reactive oxygen species and/or a decrease in intracellular levels of antioxidant-related molecules such as glutathione, catalase and superoxide dismutase leads to the release of cytochrome c from mitochondria. This triggers activation of the death cascade and apoptosis. Hsp70 is a specific inhibitor of oxidative stress-mediated apoptosis [2]. The mechanisms by which hsp27 and hsp70 inhibit apoptosis remain to be elucidated but must differ since hsp27 does not inhibit oxidative stress-mediated apoptosis and hsp70 is ineffective against death receptor-mediated apoptosis. The relation between cell death and heat shock proteins is delineated in Table 2.

The tumor suppressor protein, p53, is also involved in the induction of apoptosis. Mutations in p53 have been associated with cell proliferation and malignant transformation [3, 4]. Hsp70 binds to mutant p53, but not to non-mutated p53, and enhances its stability. The hsp70 also promotes the immunogenicity of mutated p53. In women with breast cancer, circulating antibodies to p53 could be detected only in those women who possessed hsp70-p53 complexes [5]. Thus, detection of anti p53 antibodies serves as a marker for the presence of mutated p53 within the cell.

Table 2. — *Involvement of heat shock proteins in mechanisms of cell death.*

Death mechanism	Characteristics	Heat shock protein involvement
Necrosis	cell swelling and lysis, release of intracellular contents	intracellular heat shock protein-tumor antigen complexes elicit an anti-tumor immune response
Apoptosis		
a) activation of cell surface death receptors	cell shrinkage, DNA fragmentation	inhibited by hsp27
b) intracellular oxidative stress	cell shrinkage, DNA fragmentation	inhibited by hsp70

Heat shock proteins and the immune response

The heat shock proteins are among the most highly conserved proteins in evolution. They are present in every organism ranging from bacteria to plants, to man and the human, and bacterial heat shock proteins share approximately a 50% amino acid sequence homology. Despite this amino acid sequence conservation, microbial heat shock proteins are highly immunogenic in man. Under some circumstances, such as in chronic infections, immunity to conserved epitopes of the microbial heat shock proteins leads to cross-reactive autoimmunity to the human heat shock proteins [6]. Immunity to the human heat shock proteins may also arise by molecular mimicry. Heat shock protein epitopes can also be found in a variety of other proteins [7, 8]. In addition, the association of heat shock proteins with foreign antigens, such as are expressed in infected or malignantly transformed cells, can lead to development of immunity to both the foreign antigen and the associated heat shock protein following their release from the cell.

While induction of endogenous heat shock protein synthesis leads to a decrease in production of pro-inflammatory cytokines [9] cell-free or cell surface-associated hsp70 is a potent inducer of the pro-inflammatory cytokines interleukin (IL) – 1β, IL-6 and tumor necrosis factor alpha (TNF-β) [10]. The mechanism involves binding of hsp70 to a cell surface receptor (CD14) on monocytes and macrophages. This immune activation by cell-free hsp70 may represent a first line of innate immune defense. Lysis of cells and release of hsp70 may serve as an early warning that a pathogen is present and immune activation is needed.

Heat shock proteins and cancer immunity. The function of heat shock proteins as molecular chaperones assures that in malignantly transformed cells various heat shock proteins will be associated with tumor-specific antigens. When cancer cells are killed by necrosis the release of these heat shock protein-tumor antigen complexes results in elicitation of an immune response to the tumor antigen; tumor antigens that are not associated with heat shock proteins are much less immunogenic. In addition, cell surface expression of heat shock proteins on malignantly transformed cells can activate pro-inflammatory cytokine production, as described above. The presence of pre-existing immunity to heat shock proteins in some individuals may convey additional anti-tumor immune protection.

The formation of heat shock protein-tumor antigen complexes has been exploited for anti-cancer therapy [11-13]. Isolation and purification of these complexes from tumor-bearing mice and injection into the same animals has resulted in marked anti-tumor immune activation. Injection of heat shock proteins isolated from healthy animals was without effect as was immunization with just the heat shock protein-associated tumor antigen by itself. Complexes containing hsp70 and gp96 were both effective, with the gp96 complexes exhibiting the highest level of anti-tumor immunity. Trials of immunization with heat shock protein-tumor antigen complexes have begun in man [14]. It remains too early to evaluate the success of this treatment. Among other issues, the potential negative consequences of pre-existing immunity to heat shock proteins, induction of heat shock protein-specific immunity or induction of immunity to self antigens that are also associated with heat shock proteins, following injection of the complexes remain to be addressed.

Recent studies have also demonstrated that expression of hsp70 on the surface of tumor cells triggers the activation of natural killer (NK) cells [15]. Hyperthermia has been shown to induce the appearance of hsp70 on the surface of cancer cells [16] and to increase the concentration of NK cells within tumors. Thus,

heat treatment may render some tumors more immunogenic by virtue of increased hsp70 cell surface expression. In addition, if hsp70 by itself directly activates NK cells then exogenous hsp70 may enhance anti-tumor immunity.

Clearly, the induction of heat shock proteins in malignantly transformed cells is a two-headed sword. If the anti-cancer therapy (chemotherapy and/or radiation) kills cancer cells via necrosis then a prior induction of heat shock proteins may provide a specific mechanism to make the tumor antigens more immunogenetic. If, however, the anti-cancer therapy kills cancer cells via apoptosis then a prior heat shock protein induction would render the cells more resistant to destruction. Protocols to enhance both the likelihood of necrotic killing of cancer cells and intracellular heat shock protein induction would appear to optimize the likelihood of increased anti-tumor immunogenicity. Development of such protocols should be a research priority.

Heat shock proteins and genetic polymorphisms

Unlike mutations in isolated somatic cell populations, the presence of inherited polymorphisms within the coding or promoter regions of genes in every cell of an individual are common events. The polymorphism may occur either as a single nucleotide substitution or a change in the length of a repetitive polynucleotide sequence. The different alleles of a given polymorphic gene may result in variations in the rate of messenger RNA production, messenger RNA stability or the activity of the resultant protein.

The gene coding for the inducible hsp70 is polymorphic [17]. In some individuals there is an adenosine (allele A) to guanine (allele B) substitution at position 1267 in the coding region. Individuals who are homozygous for allele B (BB) comprise less than 20% of all populations studied. It has been demonstrated that hsp70 BB homozygotes produce less hsp70 messenger RNA than do individuals who are AB heterozygotes or AA homozygotes. The consequences for malignancies of being a BB individual have been explored in two studies. In women from Tunisia, homozygosity for the hsp70 B allele was found to be associated with an increased occurrence of breast cancer and non-Hodgkin's lymphoma [18]. In a recent study from my laboratory of women with gynecologic malignancies [19], 32.0% of the 22 women studied with epithelial ovarian cancer were BB homozygotes as compared to 13.1% of 191 women with no cancer and 3.1% with endometrial cancer ($p=.009$). It still remains to be determined whether the association between homozygosity in the hsp70 B allele and ovarian cancer is due to alterations in hsp70 protein production or to the linkage of the hsp70 B allele with another, as yet unidentified, gene. This remains a distinct possibility since the gene for hsp70 is located on chromosome 6 in the class III region of the major histocompatibility complex (MHC).

Hsp27 and gynecologic cancers

Unfortunately, most women with ovarian cancer are first diagnosed when their disease is at an advanced stage. This makes their survival dependent upon the capacity of chemotherapy to control cancer cell growth. Until such time as protocols for diagnosis and treatment of early stage ovarian cancer are developed, optimization of chemotherapy for late stage disease appears to be the most realistic goal to improve survival. In the largest study of hsp27 expression in women with ovarian cancer [20], 86% of the tumors were positive for hsp27 prior to treatment. There was no relation between hsp27 expression and age, FIGO stage, tumor grade or histological type. Over the ten-year follow-up study period women with stage II-IV carcinomas whose primary tumors were negative for hsp27 had a median survival time of 61 months as compared with a 22 month survival time in women whose tumor was hsp27-positive. A second study demonstrated that malignant ovarian tumors expressed increased concentrations of hsp27 as compared to benign growth and that the hsp27 level increased with the tumor stage [21]. Similar to the first study, intra-tumor hsp27 was associated with reduced survival. A third study also reported a relation between tumor hsp27 expression and a shorter survival time in stage II-IV ovarian cancer patients [22]. All the studies are thus consistent with hsp27 being protective against chemotherapeutic agents and preventing apoptosis of ovarian cancer cells. Another study, however, of 86 women with ovarian cancer failed to establish an association between hsp27 immunostaining and cancer prognosis [23].

An in vitro study of paired ovarian cell lines that were sensitive or resistant to multiple anti-cancer drugs demonstrated that heat shock proteins were expressed only in the resistant cell line. However, mild heat treatment which further induced heat shock proteins led to the acquisition of high tolerance to cisplatin and vincristine in both cell lines [24]. This confirms the protective effect of heat shock protein expression against anti-cancer chemotherapy in ovarian cancer and also suggests that heat treatment prior to chemotherapy might not be universally beneficial.

A completely different picture emerges in women with cancer of the endometrium. Hsp27 is present in endometrial tissue from healthy women. Its concentration varies during the menstrual cycle and is highest in the late proliferative phase [25]. In hyperplastic tissue the hsp27 concentration has been shown to be decreased relative to healthy endometrium [26]. In endometrial cancer, hsp27 levels are highest in differentiated and low grade tumors [27]. One study found that hsp27 was an independent indicator of endometrial cancer prognosis, with low hsp27 levels associated with tumor recurrence and mortality [28]. Whether the difference between ovarian and endometrial cancer with respect to the significance of hsp27 concentration relates to variations in immune surveillance against the two malignancies or the predominant method of cell death (necrosis vs. apoptosis) in each are interesting unexplored areas for future research.

Antibodies to self heat shock proteins could arise from exposure of the immune system to heat shock protein-tumor antigen complexes that are released from cells that underwent necrotic cell death. In women with breast cancer, overexpression of hsp27 in the tumors was associated with a poor prognosis [29]. Conversely, the occurrence of circulating IgG antibodies to hsp27 indicated a favorable outcome [30]. Importantly, antibodies to hsp27 were not seen in healthy women. To ascertain whether heat shock protein immunity also occurred in response to gynecologic malignancies, we obtained serum and endocervical samples from women with various gynecologic malignancies and tested for IgG and IgA antibodies to hsp27, hsp70 and hsp90. The results of both studies have been published [31, 32]. Cervical IgA antibodies to hsp27 were not detected by an ELISA assay in any of 25 women with benign diagnoses or in 46 healthy women. In marked contrast, 85.7% of 21 women with untreated endometrial cancer and 41.1% of 17 endometrial cancer patients following treatment were cervical IgA anti-hsp27 positive. In women with ovarian cancer, 77.8% of seven women tested prior to treatment and 75.0% of 24 women evaluated after treatment were hsp27 IgA positive. In the six women with cervical cancer who were tested five were IgA anti-hsp27 positive. The data are summarized in Table 3. Cervical IgA antibodies to hsp70 were present both in some cancer patients and controls while anti-hsp90 IgA was most prevalent only in women with ovarian cancer. IgA antibodies are produced locally within the female genital tract, primarily in the endocervix. Thus, IgA antibodies in the female genital tract are largely distinct from those present in the circulation. The results suggested that a local genital tract immune response to hsp27 might be an early indicator of a gynecologic malignancy.

An evaluation of IgG antibodies in the sera of gynecologic cancer patients and controls revealed a similar strong association between antibodies to hsp27, but not to hsp70 or hsp60 or hsp90, and gynecologic malignancies. However, unlike the complete absence of cervical IgA antibodies in women with no malignancy, one control woman and one woman with a benign gynecologic disorder were IgG anti-hsp27 positive.

Whether cervical or serum antibodies to hsp27 are associated with a favorable prognosis in gynecologic malignancies, as was shown for breast cancer [30], remains to be determined. The presence of these antibodies, indicating an immune reaction to a self molecule, may be a marker that an immune response was also initiated against tumor antigenic peptides that were associated with hsp27 and were released from lysed cells.

Table 3. — *Cervical IgA antibodies to hsp27 in women with untreated or treated gynecologic malignancies.*

Diagnosis	Treatment	No. IgA positive / No. tested (%)
Endometrial cancer	No	18/21 (85.7)
	Yes	7/17 (41.1)
Ovarian cancer	No	7/9 (77.8)
	Yes	18/24 (75.0)
Cervical cancer	No	5/6 (83.3)
	Yes	0/4 (0)
Benign conditions		0/25 (0)
Healthy controls		0/46 (0)

Hsp70, hsp90, gp96 and gynecologic cancers

Hsp70 and hsp90 expression in endometrial carcinomas from 44 women has been investigated [33]. Hsp70 was detected in 50% of the tumors but varied greatly according to the women's age. Only 11% of tumors from premenstrual women were hsp70 positive as opposed to 60% of the postmenopausal women. The finding of hsp70 in the tumor increased as the histologic grade increased; however there was no relation between hsp70 expression and FIGO stage. Hsp70 positivity was associated with poor survival. The five-year survival rate was 90.8% in women with hsp70-negative tumors and 58.5% in those with hsp70-positive tumors. Interestingly, the over-expression of hsp70 in the endometrium has also been associated with infertility [34]. The suggests that fertility history must be taken into account when assessing endometrial hsp70 levels. Also, the increased hsp70 concentrations may retard apoptosis and foster transformed cell proliferation in the endometrium of some infertile women.

In contrast to the results with hsp70, the expression of high levels of hsp90 in the endometrial tumors was associated with a higher five-year survival rate, 100% vs. 65%. In all, 30% of the tumors had high hsp90 expression, 56% in the premenopausal women and 23% of postmenopausal women. The tumors of women with high hsp90 levels were positive for estrogen receptors and were more differentiated than were the hsp90-negative tumors [33]. An earlier study also found an association between hsp90 tumor expression and a high level of estrogen receptors; hsp70 expression was associated with a loss of steroid receptor expression in the tumor [35].

In a comparative study of 30 specimens from women with cervical intraepithelial neoplasia (CIN) and 20 specimens from women with invasive squamous cell carcinoma, hsp70 expression was significantly elevated in the latter group [36]. Similar to what is seen for endometrial carcinoma, hsp70 may be involved in proliferation of cervical malignancies and its detection may have negative prognostic value.

Two studies have demonstrated that gamma irradition [37] or therapeutic doses of retinoic acid [38] increased the intracellular expression of gp96 in cervical cancer cell lines. Since gp96-tumor antigen complexes are potent anti-tumor immune system activators the effectiveness of these treatments in enhancing anti-tumor immunity may, at least in part, be due to the up-regulation of gp96.

Future prospects

Knowledge of the positive and negative actions of heat shock proteins in tumor cell survival, progression and resistance to chemo- and radiation-based therapies has led to the design of experimental protocols to harness these properties for development of innovative anti-tumor treatments. The finding that elevated intra-tumor hsp70 concentrations are central to the growth of transformed cells and the inhibition of apoptosis encouraged investigators to seek methods to inhibit intra-tumor hsp70 production. In a recent protocol, antisense hsp70 complementary DNA (cDNA) which would inhibit hsp70 gene transcription was synthesized and inserted into an adenovirus vector. When this virus was utilized to infect several breast cancer cell lines in vitro, hsp70 synthesis was uniformly inhibited and the malignantly transformed cells underwent apoptosis [39]. Further refinement of this methodology may lead to a novel anti-cancer treatment in patients resistant to conventional therapies. Perhaps inclusion of antisense hsp27 cDNA would be of added benefit.

Synthesis of hsp70 by tumor cells in vitro is also inhibited by the bioflavonoid, quercetin [40, 41]. This treatment also inhibited growth of tumor cells as well as increased their sensitivity to thermal killing.

The rapid induction of heat shock protein genes following HSF activation can also be exploited for anti-tumor therapy. A heterologous gene was synthesized in vitro by the coupling of the promoter region of the hsp70 gene to genes coding for the pro-inflammatory cytokines IL-12 and TNF-β. These constructs were inserted into adenovirus vectors and used to infect tumor cell lines in vitro as well as melanomas in mice. Following a mild hyperthermia (39-43°C) to activate the hsp70 promoter, a 10,000-fold increase in IL-12 and TNF-β expression was observed. In the melanoma-bearing mice intralesional injection led to a significant inhibition in tumor growth [42]. Thus, utilization of the heat activation properties of heat shock protein genes will allow the selective induction by heat of any desired gene after its association with the heat shock protein promoter.

Increased production of heat shock proteins may also be beneficial in selected situations. For example, heat shock protein activation just prior to necrotic cell killing of tumor cells would enhance the immunogenicity of the liberated tumor-specific antigens and aid in the elimination of residual transformed cells. A hydroxylamine derivative, Bimoclomol, has been demonstrated to act synergistically with various stresses to markedly increase the intracellular levels of hsp60, hsp70, hsp90 and gp96 [43]. The compound had no heat shock protein-inducing activity in unstressed cells. Thus, utilization of Bimoclomol, other heat shock protein inducers or even whole body or regional hyperthermia might serve as important adjuvants when the method of subsequent tumor destruction can be accurately predicted to be non-apoptotic.

Lastly, as mentioned above, purification of heat shock protein – tumor-specific antigen complexes, from individual tumors and their utilization as anti-tumor vaccines against the original tumor has shown promise in laboratory animals. Further studies appear to be necessary to ascertain the efficacy of this approach for man. Most importantly, care must be taken to assure that this type of immunization does not enhance tumor growth or increase resistance to modulation by the immune system. Another more specific approach might be to expose the tumor-derived heat shock protein-intracellular antigen complexes to autologous cytotoxic T cells in vitro, selection of those expanded T cell clones that recognize only tumor-specific antigens and then re-infusion into the patient of only those clones.

An alternate approach is to synthesize in vitro a complex composed of the gene coding for an antigen associated with malignancy coupled to a gene coding for a heat shock protein gene, and its subsequent insertion into the patient. The feasibility of this approach has been demonstrated in experimental animals. The in vitro preparation of a microbial hsp70 gene-HPV E7 DNA construct and its insertion into an adeno-associated virus was shown to be effective in eliciting both humoral and cell-mediated immunity to HPV [44]. Such preparations seem like very attractive candidates for anti-cancer DNA vaccines.

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