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Abstracts

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Detection of Heat Shock Proteins and Heat Shock Protein Antibody Complexes in Placental Tissues following Preterm or Term Birth and Intrauterine Growth Retardation Using a New Double Fluorescence Technique.

M. Ziegert[1], I. Sziller[3], H. Alexander[1], E. Brylla[3], W. Hartig[4]

[1]Division of Human Reproduction and Endocrinology, Dept. of Ob/Gyn, University of Leipzig, Germany.
[2]Dept. of Ob/Gyn, Semmelweis University Medical School, Budapest, Hungary.
[3]Institute of Anatomy, University of Leipzig, Germany.
[4]Paul Flechsig Institute for Brain Research, University of Leipzig, Germany.

Objective: Several studies have shown the association of antibodies to chlamydial or human hsp60 and PID, ectopic pregnancy, adverse IVF outcome or impaired murine embryo development. In some cases sensitization to chlamydial hsp60 may lead to an immune response against human hsp60 due to similarities in amino acid sequences between human and chlamydial hsp60. Such a cross reactivity in pregnancy might be a cause of disturbed outcome. The incidence of systemic and local immune reactivity is of interest.

Method: Therefore, the relationship between pregnancy outcome and the presence of hsp60, hsp70 and hsp90, its antibody complexes in placental tissue and circulating antibodies to heat shock proteins was evaluated. Expression of the 60kD, 70kD and 90kD heat shock proteins (hsp) in placentae from 12 women with preterm birth, 8 with intrauterine growth restriction (IUGR) and 10 with term birth, and the presence of anti-hsp antibodies (IgG), was investigated by a new carbocyanine double fluorescence technique. This technique enables concomitant detection of human anti-hsp60-, anti-hsp70-and anti-hsp90- antibodies and hsp antigen as complexes. Results were compared with microbiological findings and circulating antibodies to hsp in the serum using ELISA and PCR.

Results: Hsp60, hsp70 and hsp90 were all identified in each placental specimen examined. Hsp70-antibody complexes were detected only in 4 of the preterm birth cases. Similarly, hsp60-antibody complexes were more frequent in preterm labor (5/12)

patients than in the term birth (0/10)- or IUGR (1/8) groups. In the preterm birth group all patients with hsp60-antibody complexes were positive for hsp60 antibodies in the sera. Altogether, 7/12 cases of preterm birth were positive for serum hsp60-antibodies while only 2/8 in IUGR and 2/10 in term birth were positive. The presence of hsp70-antibody complexes in preterm labor correlated with the presence of hsp70 antibodies in the sera (4/12). In no case of term birth and IUGR could we find serum hsp70 antibodies. The occurrence of hsp70-antibody complexes was coincident in three cases with placenta displaying hsp60-antibody complexes. None of the placentae contained hsp90-antibody complexes. The local microbiological findings showed no differences between groups in the prevalence of any infection.

Conclusion: The coincidence of circulating hsp60- and hsp70-antibodies seems to be a diagnostic marker for preterm labor. The corresponding presence of hsp60- and hsp70-antibody complexes in the placenta of patients with preterm birth supports this assumption. This is the first demonstration of hsp-antibody complexes in placentae. Further studies are necessary to determine possible synergistic actions of hsp60- and hsp70-antibodies and its immune complexes with cytokine activation to better understand the immune mechanism of preterm labor.

Does Immune Response to Heat Shock Proteins in Periodontal Disease Influence Atherogenesis?

Ingrid Glurich*, Hakimuddin Sojar, Boris Albini, Alex Ho, Mohamed Zeid, Rashesh Shah, George Wick, Robert Genco and Ernesto DeNardin.

Department of Oral Biology, S.U.N.Y. at Buffalo, N.Y.

Chronic persistent infection with periodontopathic organisms results in development of periodontal disease (PD) which affects 33% of the population of western countries. Epidemiologic studies have implicated PD as a risk factor for cardiovascular disease. Heat shock proteins (hsp) are highly conserved, immunogenic stress proteins expressed

in prokaryotes and eukaryotes and share extensive sequence homology. Immune response to hsp has also been associated with atherogenesis. Periodontal pathogens elicit antibody production, including some with specificity to hsp. Studies described herein compared immune response in periodontitis and cardiovascular disease. Levels of antibody titers to *Bacteroides forsythus* and hsp in circulation of subjects with periodontal disease, cardiovascular disease, both or neither condition, were examined. Higher titers of antibodies to hsp 60 were detected in subjects with either or both conditions compared to subjects with neither. Significantly higher titers of antibodies to *B. forsythus* were detected in subjects with periodontal disease. Reactivity of antibodies in sera from subjects with periodontal disease to a 60KD hsp homolog isolated from *B. forsythus*, was demonstrated. Reactivity of antibodies from these subjects with human hsp60 was also shown. The antigenic component in immune complexes isolated from sera of these subjects reacted with antibodies specific to hsp 60. Presence of both hsp 60 and components of periodontopathic organisms along carotid arterial walls of subjects with atherosclerosis was documented by immunofluorescence microscopy. Data support the hypothesis that immune responses elicited by periodontitis may impact exaserbation of atherogenesis. (Research supported by DEO 7926 and 4898).

Reproductive Prognosis after Ectopic Pregnancy: Does Serologic Response to the Chlamydial Heat Shock Protein 60 Play a Role?

Istvan Sziller, Mathias Ziegert, Andreas Neuer, Zsolt Csapo, Zoltan Papp

Department of Obstetrics and Gynecology, Semmelweis University Medical School, Budapest, Hungary. Department of Obstetrics and Gynecology, University of Leipzig, Germany.

Several factors including maternal age, tubal adhesions, previous ectopic pregnancy, and type of surgery have been associated with fertility after ectopic pregnancy. However, data are scanty and controversial. Up to date, no study has addressed the question,

whether previous exposure of these patients to the 60 kDa chlamydial heat shock protein (CHSP-60) might influence the chances of future pregnancies.

Reproductive performance of 78 women with ectopic pregnancy who underwent salpingectomy by laparotomy between January 1989 and December 1992 was analyzed five years later by letter-interview. Probability of reproductive events was assessed by analyzing the possible effect of history of pelvic inflammatory disease, presence of histologic salpingitis in the removed Fallopian tube, serologic response to the chlamydial major outer membrane protein (anti-chlamydial immunoglobulin G), and serologic responses to synthetic peptides corresponding to major epitopes of the 60 kDa chlamydial heat shock protein (CHSP-60). Of the 78 women interviewed, 47 (60%) were eligible for further analysis. 24 (51%) of these patients desired to conceive. Probability of becoming pregnant during the five year period after surgery was 41.6% (10/24) and probability of giving birth to a child was 33% (8/24). Among patients who desired to be pregnant after first episode of ectopic pregnancy, neither histologic salpingitis in the removed Fallopian tube, nor seropositivity to the chlamydial outer membrane protein, presence of contralateral peritubal adhesions and history of PID were associated with probability of pregnancy. In contrast, patients with serologic responses to the Chlamydia trachomatis specific epitopes in the CHSP-60 molecule ($p=0.02$), and most notably those with serologic response to two epitopes spanning between amino acids 260-271 and 411-422 were less likely to conceive than those seronegative to CHSP-60 ($p=0.005$).

These data suggest that serologic responses of patients with ectopic pregnancy to certain major epitopes of CHSP-60 might be a marker of poor pregnancy outcome in the future.

Heat Shock Protein 60 Is Exposed on the Surface of *Chlamydia pneumomae* Elementary Bodies

T. Huittinen[1], K. Laitinen[1], K. Lounatmaa[2], M. Puolakkainen[3], M. Sarvas[1], M. Leinonen[1], P. Saikku[1]. [1]National Public Health Institute and [2]Institute of Dentistry and [3]Haartman Institute, Finland.

Objective: *Chlamydia pneumoniae* is a common human pathogen associated with a great variety of both acute and chronic diseases. A 60 kDa Heat shock protein (HSP60) has been shown to be associated with the immunopathologic damage in repeated and chronic chlamydial infections (1). We have shown earlier that rabbits infected *with C. pneumoniae* had high antibody titers against *C. pneumoniae* HSP60 and the antibodies were formed simultaneously with the development of arteriosclerotic changes in aortas. These animals also showed a cross-reactive response to the HSP60 protein, suggesting the possibility of an autoimmune reaction (2). In the present study, we investigated whether this protein is exposed on the surface of *C. pneumoniae* cells.

Method: Microimmunofluorescence (MIF) and immunoelectron microscope utilizing immunogold labelling technique were performed using three sera obtained from New Zealand White rabbits immunized separately with *C. pneumoniae* HSP60, *C. pneumoniae* major outer membrane protein (MOMP) and Omp2 produced as recombinant proteins in *Bacillas subtilis*.

Results: MOMP and Omp2 were not exposed on the surface of *C. pneumoniae*, also shown earlier (3). HSP60 was demonstrated on the surface of *C. pneumoniae* elementary bodies by both MIF and immunoelectorn microscopy.

Conclusions: Both MIF and immunoelectron microscopy showed that HSP60 protein is surface exposed in *C. pneumoniae* elementary bodies. Further studies are needed to elucidate the role of HSP60 proteins in the pathogenesis of *C. pneumoniae* infection.

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Presence of Anti-HSP60 Antibodies in the Seminal Fluid of Male Partners of Infertile Couples and its Relationship with Antisperm Antibodies.

Maria Isabel Camejo and Gladys Munoz. Depto. De Biologia de Organismos, Simon Bolivar University, Caracas, Venezuela.

Introduction: Spermatozoa are not produced until puberty, long after establishment of tolerance to self-antigens during the neonatal period. Therefore, sperm-specific antigens are immunogenic in men. The fact that the majority of men do not produce antisperm autoantibodies indicates the existence of mechanisms to limit immune responsiveness in the male genital tract. When these mechanisms are disturbed by different causes such as genital tract infection, inflammation or stress conditions, autoimmunity could be activated and antisperm antibodies detected. The purpose of this study was to demonstrate a possible relationship between antisperm antibodies and the presence of anti-heat shock protein 60 (HSP60) antibodies in seminal fluid.

Material and Methods: Samples from 100 men were distributed in 3 groups: Group 1 - Infertile men positive for antisperm antibodies (ASA) (n=40), Group 2 – Infertile men negative for ASA (n=40) and Group 3 –Fertile men negative for ASA (n=20). Sperm antibodies was detected by Immunobead binding. The presence of anti-HSP60 antibodies in seminal fluid (SF) was detected by ELISA using a recombinant HSP60 (Stress Gen) bound to wells of a microtitre plate. In these samples the presence of HSP60 antigen in SF was also detected by ELISA using a monoclonal antibody to HSP60 bound to wells of a microtitre plate and a polyclonal antibody to HSP60 (Stress Gen) as detecting antibody.

Results: 42.5% of samples from men autoimmune to sperm antigens (Group I) were positive for anti-HSP60 antibodies, while only 17.5% of samples from ASA negative infertile men were positive for anti-HSP60 antibodies and in the control group 10% of SF samples were positive. The statistical analysis (Fisher exact test) demonstrated that there was a significant difference among the groups positive and negative for ASA with respect the presence of anti-HSP60 antibodies (p=0.01). The comparison between negative patients and negative control subjects for ASA was less significant (p=0.05).

The detection of HSP60 antigen revealed similar results between the positive and negative group for ASA autoimmunity.

Conclusions: The presence of ASA in seminal fluid was associated with the presence of IgA anti-HSP60 antibodies as well as that of HSP60 antigen. This last relationship had been demonstrated in our previous work. Possibly this double association in autoimmune men could be attributed to immune activation, that means not only heat shock protein expression but also anti-HSP60 antibodies. Heat shock proteins are abundant components of human spermatozoa. An immune response against sperm antigens therefore should include a reaction against HSP antigens, which could explain this association. It should be interesting to investigate if the IgA anti-HSP antibodies detected react with antigens on the sperm surface and by this way contribute to the immune mechanism involved in genital tract infection and autoimmune reactions.

Circulating Antibodies to Chlamydia trachomatis and the 10 kDa and 60 kDa Heat Shock Proteins in Women with Tubal Occlusion +/- Hydrosalpinx

Spandorfer S[1]., Neuer A[2]., Liu H-C[1]., Rosenwaks Z[1]., Witkin, S.S[2].

Center for Reproductive Medicine and Infertility[1], Division of Immunology and Infectious Diseases[2], Department of OB/GYN, Cornell University Medical College, New York, New York, USA

Introduction: Cervical IgA antibodies to C. trachomatis surface components (Ct), the 10kD heat shock protein (hsp10) and the 60kD heat shock protein (hsp60) are all associated with poor IVF outcome. Since women with tubal occlusion have an increased prevalence of chlamydial infections, we examined the occurrence of serum IgG antibodies to Ct, hsp10 and hsp60 in women with a tubal occlusion +/- hydrosalpinx. The serum antibodies reflect a systemic process. In order to evaluate the local process, we also examined the incidence of these antibodies in hydrosalpinx fluid from women with hydrosalpinges.

Materials and Methods: Sera were obtained from three groups of women undergoing embryo transfers for their initial IVF cycle: 50 women whose male partners were infertile, 58 women with tubal occlusion but no hydrosalpinx and 39 women with tubal occlusions plus hydrosalpinx. In addition, hydrosalpinx fluid (HF) was aseptically collected from 16 women undergoing laparoscopy. Samples were assayed for IgG antibodies to Ct by commercial ELISA (SeroCt, Savyon), and for antibodies to chlamydial hsp10 and human hsp60 by ELISA using purified recombinant chlamydial hsp10 and human hsp60.

Results: Clinical pregnancies were documented in 68% of the women with male factor infertility. This was higher than the 43.1% rate in women with tubal occlusions ($p=0.04$) and the 41% rate in women with hydrosalpinx ($p=0.02$). There was no relation between cause of infertility and number of oocytes retrieved or fertilized. Serum Ct antibodies were present in only 1(2%) women with male factor infertility as opposed to 15 (25.9) women with tubal occlusion ($p=0.003$) and 13 (33%) with hydrosalpinx ($p<0.0001$). Serum antibodies to hsp10 were more prevalent in women with hydrosalpinx (46.8%) than in women with male factor infertility ($p<0.0001$, 6%) or tubal occlusion ($p=0.0009$, 15.5%). Serum Hsp60 antibodies were equally more prevalent in women with tubal occlusion (41.4%) or hydrosalpinx (46.8%) than in women with male factor infertility ($p<0.0002$). Hsp60 was more prevalent in the serum of those women positive for hsp10 ($p=0.02$) or Ct ($p=0.04$) antibodies than in women lacking these antibodies. Unlike in previous studies measuring cervical IgA antibodies, there was no relation between serum antibodies to Ct, hsp10 or hsp60 and IVF outcome. These antiboidies were found in the hydrosalpinx fluid as well: Human hsp60 IgG (37.5%; 6/16), Chlamydia Hsp10 IgG (56.3%; 9/16), Chlamydia IgG (12.5%; 2/16), Human hsp60 IgA (12.5%; 2/16), Chlamydia hsp10 IgA (6.3%; 1/16), Chlamydia IgA (31.3%; 5/16).

Conclusion: Women with tubal infertility have a reduced pregnancy rate after IVF as compared to couples with male factor infertility. Infection with C. trachomatis was associated with tubal infertility and immunity to the human hsp60. Immunity to the chlamydial hsp10 was most prevalent in tubal infertility patients with hydrosalpinges

NOVEL PATHWAYS UNDERLYING *BORDETELLA PERTUSSIS*-INDUCED APOPTOSIS IN HUMAN MONOCYTES: role of ATP, mitochondria and heat shock protein 70

[1]Maria Bachelet, [2]Elisabeth Njamkepo, [3]Marie-Jeanne Richard, [1]Dominique François, [2]Nicole Guiso, [1]Barbara S. Polla

1. Laboratoire de Biologie, Physiologie et Pathologie de la Respiration, UFR Cochin Port-Royal, Paris, France
2. Laboratoire des *Bordetella*, Institut Pasteur, Paris, France
3. GREPO, Laboratoire de Biochimie C, Grenoble, France

Bordetella pertussis, the bacterial causing whooping cough in humans, is known to induce apoptosis in macrophages through an unknown mechanism involving the expression of a cyclic AMP generating toxin secreted by the bacterium: adenylate cyclase-hemolysin (AC-Hly). We previously observed inhibitory effects of AC-Hly on the expression of heat shock protein 70 and in the release of tumor necrosis factor α (TNFα) or reactive oxygen species (ROS), ruling out the participation of either TNFα or ROS in the mechanism underlying *B. pertussis*-induced apoptotic cell death. Using the fluorescent probe JC-1, we measured the transmembrane mitochondrial potential ($\Delta\Psi m$), which is the driving force for ATP synthesis by mitochondria, and whose disruption represents an early event during the apoptotic cascade. We observed that in human monocytes infected by the bacterium or exposed to purified AC-Hly, the cyclic AMP increase was paralleled by a moderate fall in ATP. A rapid decrease in $\Delta\Psi m$ followed these events. Ineffectiveness of the antioxidant N-acetylcysteine to protect monocytes from $\Delta\Psi m$ disruption induced by the bacterium, further suggests that the *B. pertussis* apoptotic pathway is not under oxidative control. Reduced levels of the anti-apoptotic Hsp70 and decreased ATP may synergize with $\Delta\Psi m$ disruption and contribute to the *B. pertussis*-induced apoptotic cell death.

Effect of Antigen Source on the Measurement of IgG Antibody Responses to the 60kDa Chlamydia Heat Shock Protein (CHSP60)

R.W. Peeling, H. Wood, D. Mabey
National Laboratory for STD, Laboratory Centre for Disease Control, Winnipeg, Canada

Objective

The association of antibody response to the 60 kDa chlamydial heat shock protein (CHSP60) with the development of adverse sequelae following ocular and genital chlamydial infection has been documented by many studies. In our studies, CHSP60 antibody response was observed in 70-90% of women with tubal occlusion but in only 32% of individuals with scarring trachoma. Although the reason for this difference may be complex, one reason may be that a fusion protein cloned from serovar L2 was used as antigen for both studies. Genital chlamydial infections are commonly caused by serovars D and E, which are antigenically similar to serovar L2, whereas the predominant serotype in the trachoma population studied is serovar A. In this study, we determined whether the source of CHSP60 antigen had any effect on the measurement of IgG antibody response in an enzyme immunoassay

Material and Methods

CHSP60-A: CHSP60 from *C. trachomatis* serovar A was cloned into pTA571 and transfected into E. coli JM109. CHSP60 was purified by polyacrylamide electrophoresis and electroelution following treatment of the gel with a CuCl solution. CHSP60-L2 (a gift of Dr. R. Stephens) was cloned from *C. trachomatis* serovar L2 and expressed as a fusion protein with glutathione S-transferase.

CHSP60 fragments (amino acid 80-277) were cloned from *C. trachomatis* serovar A and D and *C. pneumoniae* AR-39 and expressed in E. coli using the New England Biolabs IMPACT-I system. This 21 kDa CHSP60 fragment encompasses epitope 260-271found to be immunodominent in chlamydial infection in several studies and homologous to human HSP60. Serum IgG antibody reactivity against this recombinant protein fragment was determined in an enzyme immunoassay format using 100ng of protein per well and 1:500 dilution of a randomly selected subset of sera from a previously published trachoma study and sera from 50 individuals with no detectable antibody by the micro-immunofluorescence assay (seronegative group). The threshold for a positive response was determined from the mean and 3 standard deviations from the mean response of the seronegative group.

Results

Of 48 sera tested against the entire CHSP60, 12 had a positive IgG response against both CHSP60-L2 and CHSP60-A and 31 had a negative response against both antigens. One serum gave a positive response against CHSP60-L2 only and 4 gave a positive response against CHSP6-A only ($p<.001$).

Of the 44 sera tested against the CHSP60 fragments, the number (%) of sera with positive IgG antibody responses against each fragment was as follows:

Source of Antigen:	CHSP60-L2	SeroA	SeroD	*C.pneumoniae*
cases (with scarring), n=22	7 (32%)	15 (68%)	16 (73%)	13 (59%)
controls (no scarring), n=22	3 (14%)	16 (73%)	9 (41%)	16 (73%)

Conclusion: Although CHSP60 is highly conserved amongst chlamydia species, the IgG antibody reactivity of these sera appeared to be different between antigen preparations with serovar A as the cloning source compared to those using serovar L2 or D for both the entire protein and the amino acid 80-277 fragment. The use of *C. trachomatis* serovar A as an antigen source not only increased the number of positive IgG responses in the cases but also in the controls. The fragment from serovar D appeared to display different epitopes on its surface than the fragment from serovar A and it is unclear at present which epitopes may be relevant to the development of immunopathology in chlamydial infections. Further studies are needed to determine the choice of antigen and antigen source appropriate for CHSP60 assays.

Humoral immunologic response to *Chlamydia trachomatis* 57kDa heat shock protein in female mice with chronic urogenital *Chlamydia trachomatis* infection

MJ Borrego[1], V Fuentes[2], E Bissac[2], F Eb[2], MA Catry[1], J Orfila[2]

[1]Centro de Bacteriologia, Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida Padre Cruz, 1699 Lisboa codex - Portugal; [2]Laboratoires de Bactériologie et d'Immunologie, Faculté de Médecine, 80036 Amiens Cedex 1 - France

The mechanism by which *Chlamydia* (*C.*) cause inflammation and pathological changes is mostly unknown. For the trachoma and pelvic inflammatory disease it has been proposed that repeated episodes of *C. trachomatis* (*C.t.*)infection or a chronic *C.t.* infection state could be responsible for an immunological response leading to scarring. *C.t.* DNA can be detected in biological samples where viable bacteria can no longer be detected suggesting that a state of persistent infection can occur *in vivo*: the usual life cycle of *C.t.* would no longer happen, but there would be a continuous release of antigenic components stimulating the immune response[1]. Fibrosis would develop many years after the original infection and represent a hypersensitivity reaction rather than direct effect of the organism itself[2]. This delayed hypersensitivity has been related to the 57kDa *C.t.* heat shock protein (C-HSP60) and various animal models have been used for the study of this phenomenon.

Objective: 1 - to study the humoral immune response to peptide sequences specific of the C-HSP60, peptide sequences specific of mouse-HSP60 and peptide sequences common to mouse and *C.t.* HSP60, in mice of different haplotypes and chronically infected by a *C.t.* E strain. 2 - to relate the serological response to HSP60 with the histopathological changes occurring in the fallopian tubes of *C.t.* infected animals.

Materials and methods: C3H/He ($H\text{-}2^k$) and C57BL/6 ($H\text{-}2^b$) mice were first treated by progesterone and then inoculated with *C.t.* in the uterus, monthly (6 months), by vaginal route. Chronic infection was determined by serological reaction (immunofluorescence) to *C.t.* and by PCR-Amplicor COBAS™ and cell culture performed on fallopian tube samples. Peptide sequences of 10 amino acids were chosen through *EMBL* based upon peptide specificity to mouse (T-10-F peptide), *C.t.* (Y-10-Q peptide) or both (E-10-A peptide) and synthesized by *Neosystem* (Strasbourg, France). IgG and IgM antibodies to E-10-A peptide were detected by ELISA. This technique was also applied to the detection of IgG antibodies to Y-10-Q and T-10-F. Spleen cells producing IgA, IgG, IgG1, IgG2a and IgM to E-10-A peptide were quantified by ELISPOT.

Results: *C.t.* is no longer detected by culture one month after each re-inoculation by the bacteria. However a strong serological response (1:256/512) is determined and *C.t.* DNA is detected by PCR during the 6 months study period. These observations argue in favor of a chronic *Chlamydia* infection state. C3H/He *C.t.* infected mice exhibit a IgG response to E-10-A peptide since the first inoculation; this reaction decreases to a minimum after the first re-infection episode but strongly increases with the successive infection episodes. The number of IgG1 producing cells increases through re-infection episodes. C3H/He mice exhibit an IgM response to the peptide confirmed by the number of spleen cells producing IgM specific to the E-10-A peptide. The IgA responses to E-10-A are irrelevant. In these mice IgG antibodies to Y-10-Q reach important levels just after the first and fifth inoculation. The production of IgG antibodies that recognize T-10-F in C3H/He mice is very similar to the described response concerning the E-10-A peptide. IgG of C57BL/6 infected mice recognize E-10-A peptide after 3 inoculations of strain E but they recognize the T-10-F peptide the whole time. No particular IgG reaction is observed to the Y-10-Q peptide in C57BL/6 mice. Excluding 5 days after the first inoculation, neither C3H/He or C57BL/6 *C.t.* infected mice exhibited histopathological changes of the fallopian tubes,

Conclusions: C3H/He mice may react to C-HSP60 through an autoimmune mechanism, as the evolution of the humoral responses to amino acid sequences of HSP60 that are common to C-HSP60 and mice-HSP60, obtained through *C.t.* reinfection episodes are similar and important. HSP60 seems to stimulate a Th2 reaction as IgG1 producing cells specific of E-10-A can be observed; reinfection contributes to this phenomenon as the number of IgG1 producing cells increases when C3H/He animals are reinoculated. The production of IgM recognizing E-10-A peptide can argue in favor of a continuos release of *C.t.* antigens, namely the C-HSP60 during *C.t.* chronic infection that would function to the immune system as continuous stimulus. C57BL/6 mice were not expected to react to HSP60; even so IgG antibody production testify that reinfection can stimulate the immune reaction to C-HSP60 meaning that the relation between this response and haplotype is not strict. No relation could be established between the reaction to C-HSP60 and tubal histopathological changes. Seemingly the *C.t.* genotype involved or the dose of viable particles would play a role on the evolution of *C.t.* tubal infections.

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SURGICAL STRESS AND THE HEAT SHOCK RESPONSE: in vivo models of stress conditioning.

Perdrizet GA, and Rewinski MJ. Emergency Medicine/Trauma Program & Surgical Research, Hartford Hospital, Hartford, CT and University of Connecticut Health Center, Farmington, CT

Introduction: All forms of surgical therapy are stressful and injurious. The majority of surgical procedures is performed in an elective fashion and thus provides the physician with an opportunity to pre-operatively condition the patient to maximize outcome. No pre-operative protocols exist which take advantage of intrinsic cellular mechanisms to provide cytoprotection against surgically induced ischemia and reperfusion injury. We have successfully protected complex tissues and organs in the whole animal by heat shock (42.5°C, 15 min.) and recovery (37°C, 6-8 hr.) prior to a surgically induced ischemia and reperfusion insult. Cytoprotection is achieved via a complex adaptation in cellular metabolism analogous to that seen in thermotolerance phenomena. A dominant metabolic change associated with hyperthermia-induced cytoprotection is the increased expression of the heat shock genes. The use of heat shock to protect tissues and organs against ischemic injury is an example of cross-protection which we have previously designated *stress conditioning (SC).*[1]

Models & data: We have used SC to provide protection in diverse animal models of surgically induced ischemia and reperfusion.[1] **1.)** Control rat renal transplants failed to function following 48 hr. of cold ischemia (0/7 survival), while SC kidneys provided life sustaining function (4/5 survival, $p<0.002$). This protection is associated with a reduction in renal vascular resistance and preservation of microvasculature.[2] **2.)** Control pig kidney transplants showed partial function (4/8 survival) following 90 min. of warm ischemia and 24 hr. of cold ischemia, while SC kidneys always functioned (8/8 survival, $p<0.04$). **3.)** Control rat pedicled-skin flap length averaged 0.55cm longer than that predicted by fluorescein perfusion ($n=14$), while SC skin flaps averaged 2.02cm longer than predicted ($p< 0.001$, $n=10$). **4.)** Control rabbits experience 88% incidence of paralysis (7/8) following spinal cord ischemia induced by 20 min. of aortic occlusion, while SC animals never became paralyzed (0/9, $p< 0.001$).[3]

Summary: In each of the above models a positive temporal association was made between the functional protection and enhanced expression of inducible HSP70. The effects of the SC protocol on acute inflammation and microvascular physiology in these complex models will be discussed. The results from these diverse translational models will be integrated to present a unified theory of stress conditioning and cytoprotection.

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DETECTION OF *CHLAMYDIA PNEUMONIAE* REACTIVE T LYMPHOCYTES IN HUMAN ATHEROSCLEROTIC PLAQUES OF CAROTID ARTERY

Mosorin M, Surcel H-M, Laurila A, Lehtinen M, Karttunen R, Juvonen J, Paavonen J*, Morrison RP, Saikku P, Juvonen T

Department of Surgery, Oulu University Hospital (MM and TJ), National Public Health Institute, Department of Oulu (AL, PS and H-MS) and Department of Microbiology, University of Oulu (RK), and Department of Infectious Disease Epidemiology, Helsinki (ML), Department of Obstetrics and Gynecology, University of Helsinki (JP), Department of Internal Medicine, Kainuu Central Hospital, Kajaani (JJ), Finland and Department of Microbiology, Montana State University, Bozeman, Montana, USA (RPM).

Background: Linkage between *Chlamydia pneumoniae* infection and atherosclerosis has been confirmed in several studies, but the precise role of this organism in the disease process is not known. We studied the relationship and reactivity of T lymphocytes of human carotid plaques to *C. pneumoniae* antigens.

Methods and Results: Tissue specimens were obtained from 17 patients who underwent carotid endarterectomy. Immunohistological staining and/or *in situ* hybridization revealed presence of *C. pneumoniae* in 64% (11/17) of the cases. Inflammatory infiltration seen in the vessel walls consisted mainly of CD45RO+ T memory lymphocytes (median 80%; range 50-90) while CD20+ B cells and monocytes were in minor proportion. *In vivo* activated T lymphocytes were propagated from the specimens with interleukin-2 and the antigen specificity of the established T cell lines was analyzed against *C.pneumoniae* elementary body antigen. T cell lines were established from all 17 carotid tissues but none of the control specimens of ascending aorta. *C.pneumoniae* was recognized as a specific T cell stimulating antigen by 7 of 17 (41%) cases. Further analyses of the *C.pneumoniae* reactive T cell lines showed that chlamydial HSP60 induced specific proliferation in 5 of 7 (71%) cases and revealed a general T cell receptor-binding motif in human HSP60.

Conclusions *C.pneumoniae* was identified as a specific microbial antigen recognized by 41% of T cell lines propagated from *in vivo* activated plaque T cells. Our results show that cell-mediated immunity to *Chlamydia* may play a role in atherosclerotic process, and this response may involve autoimmunity.

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HUMORAL IMMUNE RESPONSE TO *Chlamydia trachomatis* 60kDa HEAT-SHOCK PROTEIN IN RUSSIAN WOMEN WITH ECTOPIC PREGNANCIES AND SPONTANEOUS ABORTIONS

Pr. J. Orfila[1], Pr. B. S. Pedersen[2], F. Betsou[1], C. Chaigneau[1], J. M. Sueur[1]

[1] Biobanque de Picardie, 16 rue Fernel, 80000 Amiens, France

[2] Dept. of Obstetrics, Aker Hospital, University of Oslo, Norway

The 60kDa heat-shock protein (HSP60) of *Chlamydia trachomatis* is thought to mediate the immunopathology that follows chlamydial infection (1). Antibodies to HSP60 are considered to be related to chronicity of inflammation and not only to chlamydial infection. The aim of this study was to investigate whether humoral immune response to *C. trachomatis* HSP60 is associated with ectopic pregnancy and/or spontaneous abortion in a high risk population, by testing sera from women from Leningrad.

The study population consisted of 457 women attending the department of gynecology and obstetrics, Leningrad Regional Hospital, St Petersbourg, Russia. Three groups are compared: a. 218 normal pregnant women (**N.P.**), b. 127 women with a history of >3 consecutive spontaneous abortions (**S.A.**), c. 112 women with ectopic pregnancies (**E.P.**). The MIF assay of Wang and Grayston (2), was used to measure *C. trachomatis, C. pneumoniae* and *C. psittaci* antibody titers. Antibody serologic response to chlamydial HSP60 was determined by a commercial ELISA assay using recombinant chlamydial HSP60 protein (International Microbio, France).

Significant antibody titers against recombinant chlamydial HSP60 were found, not only in the S.P. and E.P. groups, but also in the N.P. group. The russian « N.P. » group was maintained as the control group, as the frequency of positive anti-HSP60 serologies varies significantly with the type of population studied.

Surprisingly, there is a difference between the mean O.D.450 of the MIF IgG and IgA nagative patients (M1=0,226+/-0,036) and the mean O.D.450 of the MIF IgG and/or IgA positive patients (M2=0,278+/-0,031). Women who have been in contact with *Chlamydia*, either in a current or a past genital or respiratory infection, have higher serum anti-HSP60 antibody titers than women who have presumably never met *Chlamydia* ($p<0,08$). Therefore those two groups were studied separately.

1. MIF-negative group: there was no significant difference between the N.P. and the S.A. groups. Surprisingly, the mean anti-HSP60 O.D.450 value was significantly higher for the women with N. P. (m=0,257+/-0,053) compared to women with E.P. (m=0,199) ($p<0,03$). Furthermore, no significant difference was observed between the women with N.P., E.P. or S.A., concerning the percentage of significantly high anti- HSP60 O.D.450 values (values exceeding the mean + 2 S.D. for each group of study considered; m(MIF-)+2S.D.=0,405).

2. MIF-positive group: both the women with S.A. (m=0,313+/-0,068) and those with E.P. (m=0,310+/-0,059) had higher anti-hsp60 O.D.450 values than the control group of women with N.P. (m=0,235+/-0,043), with $p<0,06$ and $p<0,05$ respectively.
Furthermore, the assembled N.P. and S.A. group had a significantly lower percentage(6% and 8% respectively) of high O.D.450 values (superior to m(MIF+)+2S.D.=0,59) compared to the E.P. group (19%).
In the present study, we show that, in spite of a high frequency of positive anti-HSP60 serologies in a risk population, both women with spontaneous abortions and with ectopic pregnancies have higher anti-HSP60 O.D.450 values than the -already- high values of the control women with normal pregnancies. Our results suggest that when high anti-HSP60 titers are associated to current or past chlamydial infections, they are possibly implicated in adverse pregnancy outcome. However, high anti-HSP60 titers may be considered as a negative predictive factor, only for the risk of ectopic pregnancy.
At the individual level, our results suggest again that an eventual very high anti-HSP60 titer, has no particular predictive value, in the case of women who are serologically negative for *Chlamydia trachomatis*. However, in the case of women who are serologically positive for *Chlamydia*, a high anti-HSP60 value may serve as a marker for an increased risk of ectopic pregnancy ($p<0,001$).

Screening programs for the prevalence of *C. trachomatis* and follow-up of positive patients are therefore indicated to reduce reproductive failure due to, at least, ectopic pregnancies, in the risk population under study.

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Patterns of seroreactivity to *Chlamydia trachomatis* Hsp10, Hsp60, and MOMP among women with tubal factor infertility, women with active infections, or healthy uninfected women

David LaVerda[1], Lisa N. Albanese[1], Paul E. Ruther[2], Kevin A. Ault[2], Richard P. Morrison[3], and Gerald I. Byrne

[1]Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, 1300 University Avenue, Madison, WI, 53706

[2]Department of Obstetrics and Gynecology, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA, 52242

[3]Department of Microbiology, Montana State University, 109 Lewis Hall, Bozeman, MT, 59717

Immunopathological sequelae of ascending *Chlamydia trachomatis* genital tract infection in women can result in severe consequences including tubal factor infertility (TFI). Through studies examining associations of immunoreactivity and disease severity, it has been proposed that the immune response to the chlamydial heat shock protein Hsp60 may participate in the development of chronic inflammation that leads to tissue damage. However, there have been few studies that investigate other antigens which may also associate with immune pathology. The recently purified chlamydial Hsp10 is a good candidate for investigation because homologues in other species have been shown to elicit delayed hypersensitivity responses. To assess the antigenicity of chlamydial Hsp10 and to examine the frequency and magnitude of immunoreactivity among patients with differing levels of disease severity, we conducted serological ELISAs against a panel of pertinent chlamydial antigens including Hsp10, Hsp60, MOMP, and whole *C. trachomatis* EBs. Patterns of seroreactivity were then determined among healthy non-infected women, women with current infection, and women with tubal infertility. Significant differences in reactivity between the populations were determined by analysis of variance with Bonferroni post-tests or by Fisher's exact test where appropriate. Both women with active infections and women with tubal factor infertility (TFI) recognized all three antigens more frequently than healthy uninfected women. Of more

importance, those women with TFI recognized Hsp10 and Hsp60, but not MOMP, more frequently than the actively infected women. A similar pattern was seen when the amount of antigen-specific antibody present in the serum of these women was measured. Thus, women with TFI recognize Hsp10 more often and to a greater degree than either the actively infected women or the healthy uninfected women. An interesting observation was made when each patient group was subdivided on the basis of seroreactivity to whole chlamydiae, and the EB+ group was examined separately from the EB- group. Associations between Hsp10 seropositivity and TFI were enhanced in the EB+ subgroup while associations among the EB- subgroup became insignificantly different from the corresponding actively infected group. Furthermore, neither Hsp60 nor MOMP responses were significantly greater than their respective actively infected subgroups regardless of EB seropositivity. Thus among those women with significantly high chlamydial titres, Hsp10 reactivity correlated better with TFI than did Hsp60 or MOMP reactivity. These findings support the hypothesis that immunological exposure to *C. trachomatis* heat shock proteins is associated with tubal factor infertility and identifies Hsp10 as an antigen recognized by a significant proportion of women with tubal factor infertility. Like has been proposed for Hsp60, Hsp10 reactivity may also contribute to the immunopathological manifestations of severe upper genital tract complications of chlamydial disease in women. Interestingly, among women seropositive to EBs, Hsp10 reactivity increases between acutely infected women and women with TFI, but Hsp60 or MOMP reactivity does not change significantly. This study indicates that further research is warranted to more precisely define the potential contribution of Hsp10 and other conserved chlamydial antigens to the immunopathological process.

Heat shock proteins and cancer immunotherapy

Sreyashi Basu

Center for Immunotherapy of Cancer and Infectious Diseases
University Of Connecticut Health Center
Farmington, CT 06030
Tel: 860 679 4444, Fax: 860 679 4365

Heat shock protein peptide complexes derived from cancer or virally infected cells are efficient in eliciting immune response against the cells from which the HSPs are isolated. The immunogenicity of HSP preparations is due to the immunogenic peptides chaperoned by the HSP. We have shown earlier that the HSP-peptide complexes are taken up by a subset of antigen presenting cells (APCs) in vitro, and the peptides are re-presented in association with the MHC I molecules of the APCs to CD8+ T cells. This unique property of delivering exogenous antigens into MHC I antigen presentation pathway has also been

shown to be effective in "cross priming". We report that the re-presentation of HSP- peptide complex involves processing of the antigen and its presentation by MHC I as well as MHC II molecules of the APCs. We also report that murine HSPs stimulate peritoneal Mac1 (CD11b)+ cells from different strains of mice to secrete a spectrum of cytokines in vitro. The different hsps tested were observed to mediate distinctive effects with respect to cytokine release. The activation of Mac1 + cells by gp96 is accompanied by enhanced expression of costimulatory molecules such as B7-1, B7-2, CD40 and significantly higher expression of MHCII on the cell surface. APCs such as macrophages and dendritic cells are key components to the innate and adaptive immune responses. This non-specific arm of hsps may be critical in the activation of APCs (which are otherwise quiescent) and in the recruitment of other effector cells for the initiation of adaptive immune response during hsp vaccination.

CIRCULATING ANTIBODIES TO HEAT SHOCK PROTEINS IN SERA OF WOMEN WITH GYNECOLOGICAL CANCERS

Irina Korneeva, Ann Marie Bongiovanni, Monica Girotra, Tom Caputo and Steven S. Witkin
Divisions of Immunology and Infectious Diseases and Gynecological Oncology, Department . of Obstetrics and Gynecology, Weill Medical College of Cornell University, New York, NY

In human females, the association between heat shock protein expression and immunity and cancer has been primarily examined in breast cancer patients. The 27 kD (hsp27), 70 kD (hsp70) and 90 kD (hsp90) heat shock proteins are all over-expressed in breast cancer cells as compared to healthy tissues or benign lesions. The presence of high levels of hsp27, hsp70 and hsp90 in a woman's breast cancer tissues were each associated with shorter disease-free survival. Autoantibodies to these heat shock proteins were also detected in breast cancer patients but, interestingly, unique correlations with survival were documented. Whereas circulating antibodies to hsp90 correlated with reduced survival and anti-hsp70 antibodies were not related to subsequent outcome, antibodies to hsp27 were associated with improved survival. Autoantibodies to heat shock proteins in female genital tract malignancies have not been previously reported and are the subject of this investigation.

Sera from women seen for possible gynecological malignancies, or returning for evaluation after surgery and/or chemotherapy for gynecological cancer, were tested for IgG antibodies to the 60 kD heat shock protein (hsp60), hsp27, hsp70 and hsp90 by ELISA, using the purified recombinant proteins (StressGen, Victoria, BC) bound to wells of a microtiter plate. A positive result (the presence of antibodies) was operationally defined as an optical density at least two standard deviations above the mean value obtained with sera from healthy women with no history of cancer.

Autoantibodies to hsp27 were detected in only 1 (3.4%) healthy control and 1 (4.3%) patient whose lesions were benign. In marked contrast, 41% of women with gynecological cancers were anti-hsp27 IgG antibody positive ($p=0.0004$ vs. benign). Similar percentages of positive patients were seen for ovarian (48.4%), endometrial (38.2%), cervical and uterine (30.0%) and other (42.1%) cancers. No differences in the occurrence of these antibodies were observed between women with active disease or those in remission.

In contrast to the results of the hsp27 antibody testing, there was no relation between circulating antibodies to hsp60 and hsp70 and gynecological malignancies. Anti-hsp60 IgG was detected in 15% of women with benign lesions, 13.8% of healthy controls and 20.2% of cancer patients. Similarly, anti-hsp70 IgG was present in 10.5% of women with a benign diagnosis, 24.1% of the controls and 23.1% of cancer patients.

Hsp90 antibody testing revealed that 10.5% of controls and 6.9% of healthy women were IgG positive. This was lower than the 22.2% of cancer patients' sera that contained these antibodies. However, the difference was not statistically significant.

The mechanism resulting in production of autoantibodies to heat shock proteins in cancer patients, especially hsp27, remains to be elucidated. Perhaps the association of heat shock proteins with immunogenic tumor-derived antigens leads to an immune response to both molecules. Heat shock protein expression on the cell surface of tumor cells has been reported. Alternatively, intracellular heat shock proteins from disrupted cancer cells may associate with the cell surface of neighboring cancer cells thereby making them accessible to the immune system. The relationship between heat shock protein autoimmunity and long-term survival in women with gynecological malignancies

is currently under investigation. In any event, the present study demonstrates that circulating antibodies to hsp27 are associated with malignancies of the female genital tract.

Fever-Range Hyperthermia Stimulates Lymphocyte Adhesion to Intra-tumoral Vessels in Models for Murine and Human Mammary Adenocarcinoma. Sharon S. Evans, Wan-Chao Wang, David M. Schleider, and Elizabeth A. Repasky. Roswell Park Cancer Institute, Buffalo, NY 14263.

Successful cancer immunotherapy is fundamentally dependent on the ability of immune effector cells to gain access to tumor tissues. Lymphocyte migration out of the blood, across the vascular endothelial cell barrier, and into tissues involves a tightly orchestrated multistep adhesion cascade. Although many tumors including breast adenocarcinomas are highly vascularized, there is frequently only limited lymphocyte infiltration in these tissues, due in part to the absence, or low-level expression of adhesion molecules on tumor vessels. In this report, we identify a novel role for fever-range hyperthermia in stimulating lymphocyte-endothelial adhesive interactions in two independent murine models of mammary adenocarcinoma. These studies were prompted by our recent finding that hyperthermia regulates adhesion both at the level of lymphocytes and the endothelium. Specifically, fever-range hyperthermia (38-41°C for 6-8 h) acts on lymphocytes *in vitro* to enhance L-selectin-mediation adhesion of these immune effector cells to vascular endothelium (*J. Immunol.*, 160:961, 1998). The L-selectin adhesion molecule functions as a gatekeeper controlling extravasation through its ability to mediate the initial contact between free flowing leukocytes and endothelial receptors. We have further determined in non-tumor-bearing animals that fever-range whole body hyperthermia (WBH) (39.5°C for 6-8 h) stimulates the function of pre-existing L-selectin ligands on specialized high endothelial venules (HEV) within lymph nodes, a primary site of L-selectin-mediated lymphocyte trafficking. WBH also increases HEV expression of ICAM-1, a second endothelial adhesion molecule required for β2-integrin-mediated leukocyte extravasation.

These results raised the possibility that hyperthermia could also regulate lymphocyte-endothelial adhesion events in tumor-bearing animals. To address this question, BALB/c mice transplanted with the syngeneic TD40 mammary adenocarcinoma were treated with WBH to examine the effects on lymphocyte migration and endothelial adhesion. A marked increase in infiltration of L-selectin$^+$ lymphocytes was observed in regional lymph nodes and tumor tissues following WBH treatment. WBH also induced the expression of L-selectin ligand (defined by reactivity with MECA-79 mAb) and ICAM-1 on tumor vessels, directly correlating with a significant enhancement of L-selectin-mediated adhesion of lymphocytes to frozen sections of tumor tissues derived from WBH-treated animals. WBH did not increase tumor vessel expression of two additional endothelial adhesion molecules, VCAM-1 and MAdCAM-1. Surprisingly, the stimulatory effects of WBH on L-selectin ligand and ICAM-1 in tumor vessels and regional lymph nodes were sustained even 10 h after cessation of heating, in sharp contrast to results obtained in non-tumor-bearing animals in which adhesion returns to normal levels 10 h post-WBH treatment. WBH also enhanced L-selectin ligand expression

on cuboidal HEV-like intra-tumoral vessels in a xenograft model in which primary human mammary adenocarcinoma was transplanted into immunocompromised SCID mice. Notably, MECA-79-reactive endothelial proteins could not be detected prior to WBH in this tumor model for human breast cancer. Taken together, these studies are the first to demonstrate that hyperthermia actively increases the expression and function of endothelial adhesion molecules in breast carcinomas. These results support the hypothesis that fever-range WBH can promote recruitment of cellular mediators of the innate and adaptive immune response to highly vascularized tumor sites and regional lymph nodes through the regulation of L-selectin- and ICAM-1-mediated leukocyte-endothelial adhesion. (Supported by DAMD17-98-1-8311)

SELECTIVE MEMBRANE-ASSOCIATED AND CYTOSOLIC EXPRESSION OF HEAT SHOCK PROTEIN 70 IN RAT LEUKEMIC BASOPHILS

[1]Maria Bachelet, [1]Evelyne Souil, [2]Françoise Marchand, [1]Anh Tuan Dinh-Xuan, [2]Anne Weyer, [1]Barbara S. Polla

1. Laboratoire de Biologie, Physiologie et Pathologie de la Respiration, UFR Cochin Port-Royal, 24 rue du Faubourg Saint-Jacques, 75014 Paris, France.
2. Unité d'Immuno-Allergie, Institut Pasteur, 28 rue du Dr. Roux, 75724, Paris, France

Besides its molecular chaperoning function, heat shock/stress proteins (HSP) exert important functions in immunology. For example, HSP expressed at the membrane of infected or tumor cells might represent targets for T lymphocytes, underlying their involvement in the host's immune responses. We evaluated constitutive and heat-induced levels of the 72kD Hsp70 in a tumor cell line: RBL-2H3 rat leukemic basophils (*Kulczycky et al. J. Exp. Med. 139: 600-616, 1974*). Using flow cytometry, we observed low levels of intracellular Hsp70 in untreated stationary RBL 2H3, but those levels were significantly higher in exponentially growing cultures. Upon heat shock, we observed a significant increase in the expression of Hsp70 that was markedly higher in growing cells, attesting for a tight cell-cycle dependent expression. Hsp70 expression was not only observed in permeabilized cells, but measurable levels of the protein were found in non-permeabilized RBL 2H3. Further experiments using immunoperoxidase techniques confirmed that, besides its normal nuclear and cytosolic

localization, a portion of Hsp70 was also present at the membrane of RBL 2H3 cells. Membrane expression of Hsp70 is a characteristic feature of a limited number of tumor cells and does not occur in many tumor cell lines as the premonocytic lymphoma U937. HSP expression at the plasma membrane of certain tumor cells was suggested to act as recognition structures for CD3 and TcR-negative NK cells. It is tempting to speculate that our data are relevant with regard to the potential use of Hsp70 in anti-cancer immunotherapy.

POLYMORPHISM IN THE GENE CODING FOR THE INDUCIBLE 70 kDa HEAT SHOCK PROTEIN (HSP70) AND OVARIAN CANCER

Jan Jeremias, Irina Korneeva, Monica Girotra, Tom Caputo and Steven S. Witkin
Dept. of Ob/Gyn, Weill Medical College of Cornell University, New York, NY

HSP70 is induced under conditions of stress and functions in the intracellular transport of proteins, antigenic presentation of peptides and prevention of protein denaturation. The gene coding for HSP70 is located on chromosome 6 in the class III region of the major histocompatibility complex (MHC), between the B and DR loci. Polymorphisms of several genes within the MHC have been linked to disease susceptibility. A polymorphism has been identified in the HSP70 gene and a specific HSP70 allele has been linked with increased susceptibility to autoimmune diseases, breast cancer and non-Hodgkin's lymphoma. In this study we evaluated the relation between HSP70 polymorphism and the occurrence of gynecological malignancies. Vaginal wash samples were obtained from 71 women with gynecological cancers and from 191 healthy women with no history of cancer. The particulate fraction was processed to obtain cellular DNA for analysis. HSP70 polymorphism is due to a single A to G transition at position 1267 in the coding region. This creates a cleavage site for Pst endonuclease. Thus PCR analysis followed by Pst treatment generates a single 1117 base pair product for allele A and fragments of 936 and 181 base pairs for allele B. The various products were visualized on 1.2% agarose gels. Similar to that seen in other studies, among the control subjects 35.6% of women were AA homozygous, 51.3% were AB heterozygous and 13.1% were BB homozygous. In all, the gene frequencies in the control subjects were 61.3% for allele A and 38.7% for allele B. A similar distribution of genotypes and allele frequencies were seen in the 30 women with endometrial cancer and the 19 women with cancers other than ovarian cancer. In marked contrast, among the 22 women with ovarian cancer 32.0% were BB homozygous

(p=0.009 vs. control subjects, relative risk 3.117) and allele B was present in these women with a gene frequency of 52.3%. Thus, there was an increased incidence of ovarian cancer in women who were homozygous for the HSP70 BB allele. It remains to be established whether this association relates to the HSP70 gene or to its linkage with another as yet unidentified MHC gene. Individuals homozygous for the HSP70 B allele have been shown to produce less HSP70 mRNA as compared to AB heterozygotes. This might reflect differences in HSP70 protein production. Since HSP70 has been shown to be important for evocation of anti-tumor immunity and, conversely, for the resistance of tumor cells to destruction, variations in HSP70 might influence susceptibility to development of ovarian cancer.

VALIDITY OF SERUM CA-125 AS A MARKER OF EPITHELIAL OVARIAN TUMORS AMONG WOMEN WITH OR WITHOUT SERUM ANTIBODIES TO *CHLAMYDIA TRACHOMATIS*

Paavonen J (1), Anttila T (2), Björge T (3), Hakama M (4), Hakulinen T (5), Koskela P (2), Lehtinen M (2), Luostarinen T (6), Pukkala E (6), Stenman U-H (7)

Department of Obstetrics and Gynecology, University of Helsinki, Finland (1); National Public Health Institute, Finland (2); Department of Gynecologic Oncology, Norwegian Radium Hospital, Oslo, Norway (3); Department of Public Health, University of Helsinki (5), School of Public Health, University of Tampere, Tampere, Finland (4); Finnish Cancer Registry (6), Helsinki, Finland; Department of Clinical Chemistry, University Hospital, Helsinki, Finland (7)

Background: The tumor marker CA125 has been studied as a screening test for epithelial ovarian cancer. The frequency of false positive results is high, and there is no evidence that CA125 screening reduces mortality.
Material and methods: We studied the interrelationships between elevated CA125 values and the presence of serum antichlamydial antibodies in a large cohort of women (N=390,000), 111 of whom developed epithelial ovarian tumors in an average of 5 years. Of the 111 tumors, 60 were malignant and 51 were semimalignant. For each case, three matched controls free of cancer were selected. Serum IgG antibodies to *C. trachomatis* were determined by the microimmunofluorescence test. *C. pneumoniae* serovar K6P was used as a control antigen. Serum CA-125 levels were determined with the Immuno-1 analyzer (Bayer Diagnostics, Tarrytown, New York), and a cut-off level of 65 kU/L was used.
Results: Seropositivity for *C. trachomatis* was not associated with significant excess risk of developing serous ovarian cancer (OR for malignant tumors 1.5; 95% confidence interval, 0.4-4.9, OR for semimalignant tumors 2.2; 95% CI 0.5-10). Elevated CA125

predicted the diagnosis of serous ovarian tumors (OR for malignant tumors 6.9; 95% CI 1.4-35), but when we excluded *C. trachomatis* seropositive cases, the point estimate increased two-fold (OR for malignant tumors 13; 95% CI 2.3-76). Antibodies to *C. pneumoniae* had no such effect. The sensitivity of CA125 tended to be higher among the *C. trachomatis*-negative cases than among the *C. trachomatis*-positive cases regardless of the CA125 cut-off level (35-80 kU/L) or the lag time between serum sampling and the diagnosis of cancer.

Conclusions: We found better validity for CA125 among *C. trachomatis* seronegative cases. The presence of serum antibodies to *C. trachomatis* may be an effect modifier for the performance of CA125 in the detection of serous ovarian carcinoma.