The role of heat shock protein 72 in inflammatory diseases

PhD thesis

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INTRODUCTION

Heat shock proteins (HSPs) are highly conserved proteins throughout evolution. The heat shock protein-70 (HSP70) family proteins are molecular chaperones with an essential role in the assembly of polypeptides. They play a central role in refolding of disrupted proteins, thereby limiting cellular injury and restoring cellular function. The cellular expression of the inducible form of the HSP70 family (HSP72) is increased following various forms of stress like heat, ischemia, oxidative stress, heavy metal irons, radiation as well as exposure to various cytokines. The linkage between HSPs and apoptosis is widely studied. It is known, that the HSPs exert anti-apoptotic effects by inhibiting the release of the cytocrome c from the mitochondria. HSP72 inhibits caspase-9 and other caspases, as well as the extrinsic pathway of apoptosis. As extracellular, cell-surface molecules the play role in cell signaling pathways and regulation of the immune response. There are increasing number of data suggesting their important role in the antitumor response.

Smoking is the largest preventable health problem worldwide, causing five million deaths yearly. In respect of the lungs, the most important smoking-related diseases are chronic obstructive pulmonary disease (COPD) and lung cancer, both representing high morbidity and mortality illnesses. Increasing body of evidence indicates association between COPD, especially emphysema, and lung cancer mainly through the common risk factor cigarette smoke.

It has been shown that in response to cigarette smoke, apoptosis and necrosis of the alveolar wall cells occurs. This destruction results in progressive cell loss and airway enlargement: the prominent features of emphysema. Cigarette smoke consists of more than 4,000 compounds, so it is difficult to determine which components are the main contributors to cellular damage. Studies assessing the deleterious effects of cigarette smoke are mostly using different forms of cigarette smoke extracts. Cigarette smoke mediated oxidative stress and inflammatory events in the airway and alveolar epithelium are important processes in the pathogenesis of smoking-related pulmonary diseases. Tobacco smoke initiates apoptosis in airway epithelial cells as a result of mitochondrial damage. This effect appears to result mainly from free radical activity in tobacco smoke and not from nicotine. Examination of lung tissues from COPD patients revealed increased number of apoptotic cells as compared to normal or smokers without COPD lungs.

Steroids are commonly used drugs for many acute and chronic pulmonary inflammatory diseases, including asthma, COPD and lung cancer. The therapeutic effects of these agents have been mainly attributed to their anti-inflammatory and immunosuppressive effect. However, it is not clear yet, how steroids affect lung parenchyma or airway epithelium.

Steroids are stress hormones and during cellular stress increase in HSP72 might be necessary to elicit proper glucocorticoid action. It is well known, that a heat shock protein 90(HSP90)/HSP70-based

multiprotein chaperone machinery is necessary for the prompt function of the glucocorticoid receptor (GR). It plays an important role in the opening of the ligand-binding cleft of the GR, in the translocation to the nucleus, both in GR movement to transcription regulatory sites and in the disassembly of regulatory complexes as the hormone level declines. It also plays a critical role in stabilization of the GR to ubiquitination and proteasomal degradation. There are recent data that the initial GR interaction with HSP70 appears to be critical for the triage between HSP90 heterocomplex assembly and preservation of receptor function. It is possible that all physiologically significant actions of HSP90 require the HSP70-dependent assembly of client protein-HSP90 heterocomplexes.

In COPD patients the incidnce of **lung cancer** is also elevated. The main common patogenic factor is smoking. But the pathophisiology of the two illnesses seems to be different. In lung cancer the uncontrolled cell-growth plays the most important role, whereas in COPD chronic inflammation mediated airway destruction and apoptosis are the most characteristic features. However, significant proportion of the patients suffering from lung cancer is also COPD patient, and COPD is an important risk factor of lung cancer. Ueda and collegues analyzed if chronic inflammation caused emphysema or airway obstruction correlates with the risk of lung cancer in COPD patients. They found that even mild emphysema increases the risk of lung cancer. Houghton and collegues worked out two hypotheses. First: cigarette smoke induced chronic airway

inflammation damages protease-antiprotease balance subsequently destroying the lung alveoli On the other hand, due to inflammation neutrophil granulocytes and macrophages accumulate in the lung tissue, producing several growth factors. Resulting proliferation is typical for lung cancer cells. The fact that in COPD patients reciving inhaled steroid treatment the risk of lung cancer is lower confirms the central role of inflammation. The other possible mechanism is the response of the organism to the damage. In this case the chronic inflammation caused elevated cell loss activates the organism's response to repair the damage. Repair is done by bronchoalveolar stem cells. However, if the damage is extensive, the recruitment is also increased, which can cause uncontrolled cell growth, namely – lung cancer.

Small cell lung cancer (SCLC) is representing 15–20% of lung cancers and is responsible for nearly 200 000 deaths yearly worldwide. Despite the chemoradiosensitivity of SCLC survival is moderate due to early metastasis formation and/or recurrence of the disease. As median survival time ranges between 9–20 months, better understanding of tumor growth and new therapeutic strategies are needed to increase survival in this subgroup of lung cancer patients.

Besides environmental factors, genetic polymorphisms are also influencing the production of HSPs. Due to their highly conserved structure a relatively low degree of polymorphism is reported in HSP72 genes. The HSPA1B A(1267)G site of the coding region of

HSP72 gene is the most widely studied. In homozygous carriers of the G variants decreased HSP72 mRNA expression was measured.

Celiac disease (CD) is an immunologically mediated enteropathy of the small intestine, characterized by lifelong intolerance to gliadin and related prolamines. The essential function of the intestinal epithelium is the maintenance of a selective barrier through which nutrients and electrolytes can permeate, whereas potentially harmful agents are excluded. In CD the structure of this barrier is damaged, which leads to the leakage of cereal proteins across the intestinal epithelium. Wheat gliadin then activates both the innate and the adaptive immune system and increases the apoptosis of enterocytes.

Recent findings demonstrate that HSP72s are ligands for Toll-like receptors (TLRs), which play a crucial role in the defense mechanism of the innate immune system. HSP72 may exert immunoregulatory effects by binding especially to TLR2 and TLR4 on APCs.

AIMS

1. In our first study we examined how dexamethasone (DEX) acts on lung epithelial cells in vitro?
2. How does cigarette smoke extract (CSE) modify the apoptosis of lung epithelial cells, and how does DEX treatment change CSE induced cell damage?
3. How does the HSP72 expression change following DEX or CSE, and DEX-CSE co-treatment in lung alveolar epitehelial cells?
4. In our in vivo study, we investigated the HSP72 (HSPA1B A(1267)G) polymorphism in patient suffering from small-cell lung cancer (TNM IIIA-IV) in relation to control, healthy subjects. We examined the different polymorphisms' effect on the clinical outcome of the patients.
5. Does the HSP72 protein expression change according the HSP72 (HSPA1B A(1267)G) genotypes in the histologic sample of SCLC patient?
6. How does the expression and the localization of HSP72 change in untreated and treated coeliac patients compared to healthy subjects?
7. How does the gluten-free diet change the expression of HSP72?

PATIENTS AND METHODS

Investigating lung epithelial cells we used immortalized alveolar epitheliel cell line (A549), which are widely used for studying alveolar type-II epithelial cells.

We treated the cells with steroid (DEX) and cigarette smoke extract (CSE). We used the following groups: we maintained our cells in medium consisting steroid in different concentrations ((0; 0,1; 1; 10 μM), on the other hand we treated the cells with CSE:

We always prepeared the CSE before the experiment with a „hand made cigarette smoker machine". The CSE liquid's reproducibility was tested by mass spectrometry.

We measured the HSP72 mRNS expression using real time RT-PCR-rel. The protein expression was determined with FACS analysis. Both the rate of HSP72 positive cells and the intensity of each cells were measured. We investigated the apoptosis using Annexin V / PI assay. The HSP72 silencing transfection was made using SiPort NeoFx. Statistical analysis were made by Mann-Whitney U-test. $p < .05$ was considered to be statistically significant.

SCLC patients were studied at the Department of Pulmonology, Semmelweis University Budapest, Hungary. Total of 43 patients diagnosed with locally advanced, advanced, and metastatic disease SCLC (TNM: IIIA-IV) between March 2003 and September 2004 were selected into the analysis. During this time-period, 486 patients were diagnosed with lung cancer at our Department, including 72 SCLC patients. Only patients with advanced disease and available

histology or cytology were included into the analysis representing> 60% of all SCLC patients diagnosed during the observation period. Blood sampling was performed at different time points after diagnosis. Data on co-morbidities, smoking history, performance status, TNM stage at the time of diagnosis as well as data on chemotherapy cycles and radiotherapy, therapy associated major side effects were collected

Survival was analyzed following 60 months. All patients gave their informed consent for taking blood sample for DNA analysis according the Helsinki declaration. The prevalence of HSP72 [HSPA1B A(1267)G] genotype was obtained by studying a random, unrelated population sample of 97 healthy adult blood donors.

The HSP72 expression of the tumor cells we measured using immunohistochemistry. The relative expression of HSP72 in tumor cells was assessed (on a scale between 0–4), and expressed as percent of the expression seen in bronchial epithelial cells of the same sample (also graded 0–4).

The prevalence of HSP72 [HSPA1B A(1267)G] genotype was obtained by using PCR and RFLP.

Statistical analysis: Hardy–Weinberg equilibrium was calculated to evaluate the relationship between gene frequencies. Clinical characteristics of SCLC patients were analyzed using Cox regression analysis. Categorical data were analyzed by $\chi 2$- or Fisher's exact test. Survival of different subgroups was assessed using Kaplan–Meier analysis. $p < .05$ was considered to be statistically significant.

Duodenal biopsy samples from 16 children with untreated and 9 with treated CD were collected in the 1st Department of Pediatrics, Semmelweis University. Biopsy samples of 7 children with untreated CD were taken at the time of diagnosis, before the introduction of a glutenfree diet. From the other 9 children duodenal biopsies were obtained before (untreated CD) and 1.5 years after exclusion of gluten from the diet (treated CD). The diagnosis of CD was based on the ESPHGAN criteria. The control group consisted of 10 children who were investigated for either growth retardation or chronic diarrhea, and an upper gastrointestinal endoscopy was part of their diagnostic procedure. No significant age- or sex-related differences were observed among children with untreated CD, treated CD, and controls. Written informed consent was obtained from parents of each participant before the procedure.

We measured the HSP72 mRNS expression using real time RT-PCR. The protein expression was measured using Western-blot analysis. Localization of HSP72 in the cells was assessed using immunofluorescent staining.

Statistical analyses were made by Mann-Whitney U-test, after Shapiro-Wilk's test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Under control conditions cells proliferated, reaching about 8.55±1.1×105 final cell number/vial. Following DEX treatment no significant change in cell number was noted. In contrast, CSE treatment significantly reduced the cell number 24 h after incubation as compared to controls (C). CSE+DEX co-treatment dose-dependently and significantly increased the total cell count as compared to CSE treatment alone, reaching similar number in both DEX (10) groups.

DEX slightly decreased the number of the apoptotic cells in controls, reaching statistical significance only in the C+DEX (10) group. In steroid-naïve CSE-treated cells, apoptosis tripled as compared with the steroid naïve controls. DEX treatment significantly reduced apoptosis in all CSE-treated groups, abolishing the difference between CSE-treated and respective control groups.

HSP72 mRNA expression did not change following DEX treatment in controls. Administration of CSE alone was associated with low level of HSP72 mRNA, similar to the level observed in steroid naïve controls. In contrast, DEX treatment resulted in significant increase in HSP72 mRNA, already using 0.1 μM concentration in the presence of CSE, and further significant increase was observed using 1 μM DEX. However, no further increase was detected in the CSE+DEX (10) cells.

In steroid naïve controls HSP72 protein expression of individual epithelial cells was low, similarly to the ratio of HSP72 expressing cells. DEX treatment decreased both the ratio of HSP72 expressing cells and the cellular HSP72 content of the cells significantly in controls. In all CSE-treated groups, the ratio of HSP72-positive cells was significantly higher as compared to respective controls ($p<0.05$). DEX treatment significantly and dose-dependently increased the number of HSP72 expressing cell, with the highest ratio measured in the CSE+DEX (10) group, where nearly 80% of cells were expressing the protein. CSE treatment significantly increased the cellular HSP72 protein content in all (steroid naïve and DEX treated) groups as compared to respective controls. The mean intensity of HSP72 in HSP72-positive cells increased with increasing doses of DEX following CSE treatment, with significantly increased values in DEX (1) and DEX (10) groups compared to DEX (0) group.

Transfection with siRNA was successful in steroid naïve controls and CSE-treated cells. As previous experiments confirmed the highest HSP72 expression in CSE+DEX (10) cell, this group was chosen as the third group, to assess the effect of CSE and steroid co-treatment on cellular HSP72 expression in this experimental setting. While scr-RNA did not change cellular HSP72 protein expression, siRNA treatment resulted in significant decrease of cellular HSP72 protein under control and CSE treated conditions. Similarly, HSP72 protein expressing cell number significantly decreased in all groups following siRNA treatment. In C and CSE groups, siRNA reduced

the number of HSP72-positive cells by 80% and 60%, respectively, while it reached 75% in the CSE+DEX (10) group. The most marked decrease in HSP72 was registered in the CSE+DEX (10) group following siRNA treatment, where significant reduction in the ratio of HSP72-positive cells, and the reduction of the content of the expressing cells was observed abreast. Parallel to the decreased expression of HSP72 protein in siRNA treated cells, apoptosis significantly increased in all groups, indicating a direct link between cellular HSP72 and apoptotic cell death.

Hardy–Weinberg criterion for HSP72 polymorphism was fulfilled in both, study population and healthy controls. Allele frequency showed a nonsignificant decrease of the A allele in SCLC patients as compared to controls [OR: 0.88 (95% CI: 0.53–1.46); p =0.69). In contrast, the GG genotype occurred more frequently in SCLC patients than healthy controls but did not reach statistical significance (19% vs. 13%).

Median survival time in patients carrying the A allele [groups AA plus AG: 11.97 months, CI (95%) 6.83–17.11] significantly exceeded the median survival time observed in GG group SCLC patients [8.15 months CI (95%) 6.37–9.93, p <.05].Median survival was shorter in patients with HSP72 AA genotype as compared to AG genotype patients, however it did not reach statistical significance. This is due to one patients very short survival following diagnosis in the AAgroup (1.86 months) as it can also be seen in the confidence

interval data for this groups. All other patients in this group had a survival time of at least 6 months.

As expected, all cells showed HSP72 staining, however the relative extent of intracellular HSP72 expression did differ between epithelial and tumor cells. Analyzing the association between HSP72 protein expressions assessed by immunohistochemistry of tumor cells and HSP72 [HSPA1B A(1267)G] genotype HSP72 staining showed a significantly decreased expression in SCLC cells as compared to normal cells in tumor samples of GG genotype patients (relative staining of cancer cells to lung cells 28%). In SCLC tumor specimen of AA (relative staining of cancer cells to lung cells: 68%) and AG (relative staining of cancer cells to lung cells: 75%) genotype patients, no difference was observed. As histological analysis of HSP72 did not reveal differences in SCLC cell staining in the presence of the A allele, patients with HSP72 AA or AG genotype were analyzed as one group for survival. Survival analysis showed significantly decreased survival in GG genotype SCLCpatients.

More advanced disease at diagnosis was observed in patients with the GG genotype, as significantly more patients (62%) showed distant metastases (M1) at time of diagnosis (vs. 29% in AG and 28% in AA groups, $p < 0.05$).

Cox proportional hazard regression showed significant impact on survival according to HSP72 GG genotype, smoking intensity (defined by pack year), age, and body mass index (BMI). Gender did

not influence outcome and increase in stage (TNM IIIA, IIIB, and IV) did not increase the risk of death in our patients. Important negative prognostic factors included lower number of applied chemotherapy cycles ($p = 0.0007$) as well as the presence of obstructive lung disease ($p = 0.0125$). Other parameters, like performance status, chest radiology, endobronchial morphology, paraneoplastic syndromes as well as side effects of chemo- or radiotherapy did not differ between SCLC patients according to HSP72 genotype.

HSP72 mRNA expression was significantly increased in the duodenal mucosa of children with untreated CD as well as children with treated CD compared with that in controls ($p=0.0002$ and $p=0.023$, respectively). In the duodenal mucosa of children with treated CD, the HSP72 mRNA level was decreased in comparison to children with untreated CD ($p=0.003$).

Significantly elevated HSP72 protein levels were detected in the duodenal mucosa of children with untreated and also with treated CD compared with that in controls ($p=0.0001$ and $p=0.003$). In the duodenal mucosa of children with treated CD, HSP72 protein levels were markedly higher than in untreated CD ($p=0.002$).

In the duodenal villi of children with untreated CD, strong HSP72 staining intensity was found in the villous enterocytes and immune cells of the lamina propria compared with controls. In the duodenal villi of children with treated CD, weaker HSP72 signal was found in

villous enterocytes and immune cells of the lamina propria compared with children with untreated CD. In the normal duodenum of controls only weak HSP72 immunoreactivity was observed. HSP72 was not present at detectable levels in the nuclear fractions.

CONCLUSIONS

1. Steroid (DEX) treatment did neither affect apoptosis nor the cellular HSP72 expression of the lung alveolar epithelial cells under control conditions in vitro.
2. CSE treatment significantly decreased survival and proliferation of the alveolar epithelial cells. CSE caused serious cell damage and was associated with increased proportion of apoptotic cells. Addition of DEX significantly increased survival and proliferation by decreasing apoptosis in CSE treated cell dose-dependently.
3. Under control condition steroid treatment did not alter HSP72 expression of alveolar epithelial cells. CSE treatment significantly increased the rate of HSP72 positive cells, and the HSP72 content of the individual cells. Co-treatment with DEX and CSE increased the HSP72 expression dose-dependently. The HSP72 protein expression was significantly higher in all CSE treated groups compared to the control groups. Transfection with HSP72 silencing mRNA confirmed the central role of HSP72 in the survival imroving effect of DEX in case of CSE treatment.
4. Investigating the HSP72 (HSPA1B A(1267)G) polimorphism in SCLC patients we found that allele frequency showed a non-significant decrease of the A allele in SCLC patients as compared to controls. In contrast, the GG genotype occurred more frequently in SCLC patients than healthy controls but did

not reach statistical significance. Survival analysis showed significantly decreased survival in GG genotype SCLC patients, confirming that the absence of A allele was associated with increased mortality. More advanced disease at diagnosis was also a prognostic factor for survival, as 62% of patients with the GG genotype showed distant metastases (M1) at time of diagnosis. HSP72 (HSPA1B A(1267)G) polimorphism is an important, significant prognostic factor in SCLC patient affecting tumor progression and survival.

5. Analyzing the association between HSP72 protein expression assessed by immunohistochemistry of tumor cells and HSP72 (HSPA1B A(1267)G) genotype HSP72 staining showed a significantly decreased expression in SCLC cells as compared to normal cells in tumor samples of GG genotype patients. In SCLC tumor specimen of AA (62%) and AG (70%) genotype patients no difference was observed.
6. In the duodenum biopsy samples of both the treated and untreated patients we found significantly elevated HSP72 mRNA and protein levels compared to the controls. In coeliac patients the treatment significantly decreased the HSP72 mRNA and protein levels. We have seen similar changes analysing the HSP72 localisation. We found strong HSP72 staining intensity in the duodenum villi and also in the immun cells of the lamina propria in untreated, coeliac children compared to the controls. While in the biopsy samples of

treated coeliac patients we saw weaker staining intensity. In the sample of normal, control children we found very weak immunoreactivity.

7. The gluten-free diet significantly decrease the HSP72 expression both in the enterocytes and in the immune cells of the lamina propria.

SUMMARY

In conclusion, our data confirmed that CSE induces apoptosis and necrosis in alveolar epithelial cells. DEX reduces CSE-induced cellular damage, by decreasing apoptosis. This is the first evidence of DEX-CSE interaction showing a key role of HSP72 in alveolar epithelial cell survival. Our siRNA experiments confirmed that elevated HSP72 is essential in the observed anti-apoptotic and protective effects of DEX following CSE exposure. HSP72 might represent a new key molecule and a potential therapeutic target in smoke exposed lung cells. As millions of smokers are treated with glucocorticoids new data on cigarette smoke and glucocorticoid interaction are needed. Future experiments are necessary to evaluate the role of HSP72 in smoker and non-smoker COPD patients, especially assessing the effects on alveolar destruction.

Our results demonstrated that HSP72 (HSPA1B A(1267)G) polymorphism is a significant prognostic factor of SCLC outcome. The HSP72 (HSPA1B A(1267)G) GG variant in SCLC patients was associated with decreased HSP72 protein expression in tumor cells. Impaired cytoprotective functions and decreased anti-tumor immunity of HSP72 might be a contributing factor to more rapid tumor progression as noted in GG genotype patients by metastasis formation and shorter survival – as we could see it in our patients, even if the sample size was small. This supports the assumption that

altered expression of HSP72 might influence protection against stress factors and thereby decrease anti-tumor immunity.

The elevated level of HSP72 in the duodenal mucosa of children with CD found in our study indicates that this molecule may have a role in defense against the gliadinmediated cytotoxicity. Because of its antiapoptotic effects it may foster surveillance of the epithelial cells and help to retain their integrity, potentially diminishing villous atrophy, which is a major symptom of the disease. HSP72 as a cellular chaperone activated due to stressors may serve as a ‘‘danger signal’’ for the cells of the innate immune system to promote their protection against injury. Additional studies are needed to clarify the precise role of HSP72 in CD. Furthermore, due to the protective effects of HSP72 it could be regarded as a potential therapeutic target to treat this gastrointestinal disease.

PUBLICATION LIST

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