antibodies are of pathogenetic significance or may be used as a disease marker will have to be elucidated in future experiments.

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e-mail: **aschaefler@gmx.de**

A.E. Schäfler. Ph.Weigold

ANTIBODIES TO HUMAN HEAT SHOCK PROTEIN 60 AND MYCOBACTERIAL HEAT SHOCK PROTEIN 65 IN PATIENTS WITH CORONARY ATHEROSCLEROSIS

Department of Cardiac Surgery, University of Ulm, Germany

Objective: In patients with coronary atherosclerosis, conflicting results related to the influence of heat shock protein antibodies exist. Our aim was to determine antibodies against human HSP60 and mycobacterial HSP65 in sera of patients with coronary atherosclerosis.

Methods: Immunoblot analyses of antibodies against human HSP60 and mycobacterial HSP65 were performed in serum from 117 patients with coronary atherosclerosis and 50 patients without angiographic evidence of coronary atherosclerosis.

Results: Anti human HSP60 autoantibodies were found in 8 patients undergoing CABG (p=0,11). Anti mycobacterial HSP65 antibodies were found in six CABG patients and one patient without coronary atherosclerosis (p=0,68).

Conclusions: This result is consistent with a previous report, suggesting that antibodies against human HSP60 or mycobacterial HSP65 may not be involved in coronary atherosclerosis.

Key Words: Atherosclerosis, heat shock protein 60/65, immunoblot

1. Introduction:

In the multifactorial pathogenesis of atherosclerosis, antibodies to the 60 kD heat shock protein (HSP) class have been demonstrated. Elevated levels of mycobacterial HSP65 (mHSP65) antibodies were reported in patients with coronary atherosclerosis [1,2]. Also a strong association has been found between high anti mHSP65 levels and restenosis after PTCA [3]. Moreover mHSP65 antibody titers were higher in patients with future cardiovascular events [4].

Additionally, in patients with coronary atherosclerosis high levels of auto antibodies against human HSP60 (hHSP60) were found [5]. They are considered an independent, novel family risk factor for severe coronary atherosclerosis [6]. Furthermore levels of complement activating anti-hHSP60 antibodies are elevated in atherosclerosis related disease [7].

In contrast to these results, a previous investigation showed that serum IgG antibodies to human HSP60 are not associated with CAD [8]. The aim of the present study was therefore to determine antibodies against myocobacterial HSP65 and human HSP60 in sera of patients with and without angiographic evidence of coronary atherosclerosis.

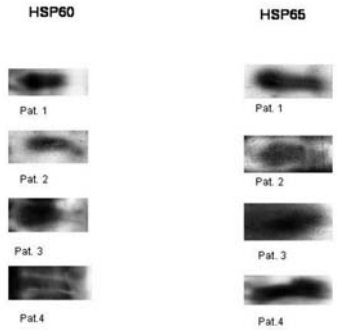

Fig. 1. Representive immunoblots of 4 patient sera with membrane bound human HSP60 and mycobacterial HSP65. Detected with horseradish peroxidase secondary AB, ECL-plus technic, exposed to hyperfilm.

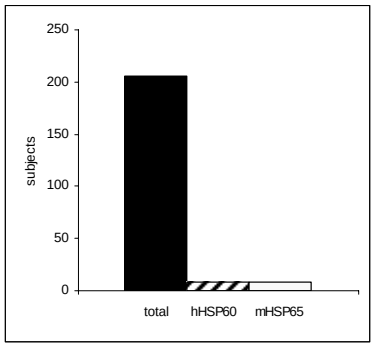

Fig. 2. Immunoblotting results from 167 subjects. 7 sera were positive for mHSP65 (p=0,68) and 8 sera positive for hHSP60 (p=0,11) each .

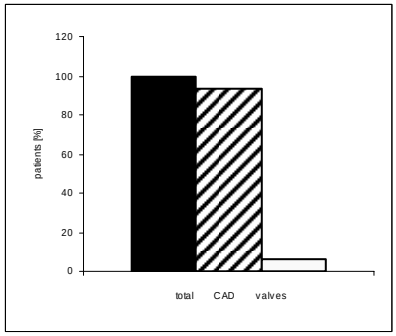

Fig. 3. Coronary artery atherosclerosis and mHSP65/hHSP60 autoantibody status. Percentage of patients positive for mHSP65 and hHSP60. 93.3 % of patients were from the coronary atherosclerosis group. 6.7 % were from the non CAD group, or the clinical control group.

2. Materials and methods:

2.1 Patients

The present study was reviewed and approved by the ethical committee of the University of Ulm. Analyses of antibody levels against human HSP60 and mycobacterial HSP65 were performed in serum from 167 patients. Serum was obtained the day of admission from all patients undergoing elective cardiac surgery for aortocoronary bypass and/or aortic respectively mitral valve replacement or repair. The clinical characteristics are given in Table 1.

2.2 Immunoblot Analysis

2.2.1 Serum Preparation

Circulating antibody concentrations were determined in serum samples obtained from all study subjects after centrifugation at 4000 rpm for 4 min in a Labofuge GL (Heraeus Sepatech, Biofuge primo R) and stored at 4 °C.

2.2.2 Membrane Preparation

The Hybond-PVDF membrane (Amersham Buchler Ltd) was preincubated in pure methanol for 10 seconds, rinsed in aqua bidest for 5 minutes and blocked for 10 minutes in blocking buffer (70% aqua bidest, 20% methanol pure, 10% tris glycerine).

2.2.3 Protein Preparation

The proteins were purchased by Stressgen. After different preparation steps, protein was transferred to the membrane. The blot was blocked 1 hour in 5 % nonfat milk solution (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 5 % nonfat milk powder, pH 7,45), incubated with the 1:10 diluted (TTBS) patient serum for 30 minutes and washed six times for five minutes in 10 ml TTBS solution. Immunodetection of the serum antibodies was carried out with a 1:10 000 diluted peroxidase conjugated anti human IgG secondary antibody (NA 933 Amersham Ltd) for 30 minutes and washed again six times for five minutes in TTBS. After incubation with 0,1 ml/cm² ECL-plus detection reagent (Amersham buchler Ltd) for 5 minutes, blots were exposed to Hyperfilm ECL (Amersham Ltd) for 10 seconds, 5 and 15 minutes.

Table 1.
Characteristics of the study subjects. ACB indicates aortocoronary bypass; MVR mitral valve replacement/repair; AVR aortic valve replacement/repair; m male; f female; y years; m male; prot. protein; conc. concentration; mg milligram; ml millilitre

group	Total	ACB	AVR/ MVR
	N	n	n
patients	167	117	50
age [y]	68.1 ± 8.9	65.7 ± 8.6	70.4 ± 9.1
sex [m/f]	119/48	93/24	26/24
prot. conc. [mg/ml]	31.4	32.6	30.1
anti-hHSP60	8	8	0
anti-mHSP65	7	6	1

2.2.4 Quantification of Immunoreactive Bands

After development the blots were scanned with a densitometer (Umax, Astra 6450, Fremont, CA; Canon, CanoScan1220 U). Bands were quantified with an analysis software (mars 98, version 1.0.1) according to the densitometric integral derived from each sample band. The integral of the density over a measured area, was taken to calculate the amount in each sample according to the known standard values. A linear relationship was found between the known antibody concentrations and the densitometric integrals. On the basis of this linear relationship anti human HSP60 and anti mycobacterial HSP65 of each serum sample was calculated ($r = 0.96$) respectively ($r = 0.85$).

2.2.5 Statistical analysis

To detect differences between groups, the Fisher Exact test was performed. The $P < 0,05$ value was considered statistically significant. The correlation between standard HSP60/65 and the densitometric integral was examined by linear regression analysis.

3. Results:

3.1 Antibody titers in sera of patients with coronary atherosclerosis

A total of 167 subjects were studies. Men constituted 71.2 % and whites 100 % of the cohort. Their ages ranged from 38-89 years for the patients.

In order to assess whether there were any changes in antibody status, immunoblot analysis was carried out from 117 patients with angiographic evidence for severe coronary atherosclerosis and 50 patients without coronary atherosclerosis.

To demonstrate antibody reaction, representative immunoblots are shown in figure 1. Visual inspection of the antibody bands shows the different band densities. Quantitative analysis revealed 8 sera positive for hHSP60 and 7 sera positive for mHSP65 from 167 subjects studied. Graphic illustration according figure 2, displays only a minority of patients positive for hHSP60/mHSP65.

If analysed according the underlying pathogenesis

graphic representation shows that the majority of patients positive for hHSP60 and mHSP65 derive from the coronary atherosclerosis group (Fig. 3). But statistical analysis shows an insignificant distribution according disease groups. Anti human HSP60 antibodies were found in eight patients (p = 0,11). All of them had coronary atherosclerosis. Anti mycobacterial HSP65 antibodies were detected in six patients from the CAD group and one patient underwent valve replacement. (p = 0,68). (Figure 2)

4. Discussion:

We investigated the association between antibody status to human HSP60 and mycobacterial HSP65 in patients with coronary atherosclerosis and patients without angiographic evidence of coronary atherosclerosis. Our findings demonstrate that almost all HSP60 and HSP65 positive sera derive from the coronary atherosclerosis group. In patients without angiographic evidence of coronary atherosclerosis only one positive immunoblotting result was obtained.

Our results support a recent study showing that antibodies against human HSP60 may not be involved in coronary atherosclerosis [8]. Rather a previous infection with chlamydia pneumoniae may in parts be associated with coronary atherosclerosis [9]. Thus, the correlation between atherosclerosis and anti HSP antibodies may derive from infection [10]. This is also supported by the finding that the correlation of anti-mycobacterial HSP65 antibodies with carotid thickening was observed in older age groups [11].

Because of the small amount of patients positive for anti hHSP60 and anti mHSP65 we couldn't show an association between HSP60/HSP65 antibody concentration and severity of coronary atherosclerosis.

Our seroepidemiological data are subject to limitations because we didn't examine antibodies to other bacterial HSP and laboratory markers of infection. In addition our study is cross sectional and natural fluctuations in antibody concentration may weaken statistical significance. Moreover, differences to other results might be explained by our immunoblotting method or study population.

In summary our results add no substantial arguments to the hypothesis of an exclusively anti human HSP60 or anti mycobacterial HSP65 associated genesis of coronary atherosclerosis. We think that the immunological reaction to infection may be one component apart from classical risk factors.

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e-mail: **aschaefler@gmx.de**