Expression of heat shock protein 72 by alveolar macrophages in hypersensitivity pneumonitis

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Racine, Claudia, Evelyne Israël-Assayag, and Yvon Cormier. Expression of heat shock protein 72 by alveolar macrophages in hypersensitivity pneumonitis. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L501–L505, 1999.—The current study was done to look at a possible role of heat shock proteins (HSPs) in hypersensitivity pneumonitis (HP). The specific aims were to determine whether there was a difference in the expression of HSP72 in alveolar macrophages (AMs) between mice challenged with HP antigen and saline-treated control mice and between AMs obtained by bronchoalveolar lavage from 18 patients with HP and 11 normal subjects. The expression of HSP72 was studied under basal conditions and under a mild heat shock. HSP72 expression by AMs in response to in vitro stimulation with Saccharopolyspora rectivirgula was lower in AMs of control mice than in those of HP animals. HSP72 was constitutively expressed in AMs of both normal and HP subjects. Densitometric ratios showed that AMs from normal subjects responded to heat shock with a 39°C-to-37°C ratio of 1.72 ± 0.18 (mean ± SE), and AMs from HP patients responded with a ratio of 1.16 ± 0.16 (P = 0.0377). This decreased induction by additional stress of AMs could lead to an altered immunoregulatory activity and account for the inflammation seen in HP.

Bronchoalveolar lavage

HEAT SHOCK PROTEINS (HSPs), also called stress proteins or chaperones, are highly conserved proteins. They are classified in different families based on their molecular weight (e.g., HSP60, HSP70, and HSP90) (26, 38). The stress response of these proteins is common to all cells studied thus far (3, 22, 25, 27, 39). HSP expression is greatly increased in response to a broad spectrum of inducers including high temperature, foreign particles, and ischemia (20).

Some studies (1, 14, 36) suggested that activation of the stress response might be caused by the accumulation of abnormal proteins. HSPs are not only important in periods of stress but are also involved in many physiological cell functions (4, 20, 30) such as the folding of newly synthesized proteins, translocation of proteins through intracellular membranes (11, 17), endocytosis, and antigen processing (28). They also participate in the removal of abnormal proteins (13).

It has been proposed that HSPs play an important role in the inflammatory process. Recent studies have demonstrated that HSPs offer cellular protection against the cytotoxic effects of interleukin (IL)-1, tumor necrosis factor-α (12, 15, 19, 23), or endotoxin (18).

Hypersensitivity pneumonitis (HP) is an interstitial pulmonary disease resulting most frequently from an allergic response to repeated exposure to a variety of organic antigens (32a). HP is initially characterized by a lymphocytic alveolitis and a granulomatous pneumonitis. Improvement or a complete reversibility occurs if the antigenic exposure is removed before permanent scarring or emphysema has developed (7).

Some studies (34, 35) have shown that HSPs are involved as a protective mechanism in acute lung injury. An important feature of the stress response is the inhibitory effect on nonstress protein gene expression. During stress, cells undergo a prioritization of gene expression characterized by the rapid expression of stress protein, whereas the expression of various nonstress proteins is transiently inhibited (26). It has been postulated that stress response-mediated inhibition of gene expression, particularly proinflammatory gene expression, may be involved in the mechanisms by which the stress response protects against lung injury (38). Moreover, the expression of HSP70 is increased in airway cells of asthmatic patients, and this is correlated with the severity of the asthma (33). Alveolar macrophages (AMs) of patients with adult respiratory distress syndrome express higher levels of HSP72 mRNA than normal subjects (22). With HP being an inflammatory disease, it is reasonable to assume that the stress response could play an important role in its pathophysiology. Because proinflammatory cytokines such as IL-1, IL-8, and tumor necrosis factor-α are increased in HP (9, 10) and because the normal function of HSP is to downregulate these cytokines (5, 29), one could hypothesize that the HSP response in HP is inadequate to control the release of these damaging cytokines and lead to the disease state of inflammation in the lung with a febrile reaction typical of HP.

The purpose of the present study was to look at the expression of HSP72 by AMs in HP. We first did studies with an animal model of HP that consists of the induction of HP by repeated intranasal instillations of Saccharopolyspora rectivirgula. The expression of HSP72 was also tested in AMs of HP patients and control subjects.

METHODS

Animal Study

Animals. Pathogen-free C57BL/6 mice were purchased from Charles River (Saint-Constant, QC).

Bacterial antigen. Crude S. rectivirgula antigen was prepared as previously described (28). The lyophilized antigen was reconstituted with pyrogen-free saline at a concentration of 5 mg/ml.
Protocol. Mice were anesthetized with isoflurane (Ohmeda Pharmaceutical Products, Mississauga, ON). The animals were separated into two groups: group 1 (n = 32) received 50 µl of saline by intranasal instillation 3 days/wk and group 2 (n = 6) was given 50 µl of S. rectivirgula antigen intranasally 3 days/wk. The animals were killed 4 days after their final saline or antigenic instillation, 6 wk after the beginning of the challenges. A different number of animals were included for each group, estimated from the results of previous studies (9, 10) with this model. The objective was to recover an adequate number of pooled lavage cells for each group.

Bronchoalveolar lavage. To harvest lung cells, the trachea was canulated with a 20-gauge plastic catheter (J ohnson & J ohnson, Arlington, NY) immediately after death by cervical dislocation. Three aliquots of 1 ml of sterile 0.9% saline solution were successively introduced into the lungs, and the fluid was gently aspirated after each injection. The recovered bronchoalveolar lavage (BAL) fluid was centrifuged, and the cells were resuspended in saline. Total cell counts were done with a hemocytometer. Cell viability was assessed by trypan blue exclusion. Total and differential counts were done with the microscope glass-cover technique (24) and Diff-Quik coloration (Baxter Diagnostics, Mississauga, ON).

Isolation of AMs. Total lavage cells were distributed in 24-wells plates (Becton Dickinson Labware, Lincoln Park, NJ) to obtain ~1 × 10⁶ macrophages/well (16, 31). Cells were allowed to adhere to plastic dishes for 1 h at 37°C and 5% CO₂ with RPMI 1640 medium, 10% fetal bovine serum, and 1% penicillin-streptomycin (GIBCO, Grand Island, NY) in a water-jacketed incubator. Nonadherent cells were washed out with Hanks' balanced salt solution.

Heat shock on AMs. Cultures of purified AMs were heat shocked in a water bath for 20 min at 42°C (12, 35). Control cells were put at 37°C for 20 min. After heat shock, a fraction of the AMs was stimulated with 25 µl (5 mg/ml) of S. rectivirgula antigen preparation. Cells were allowed to recover for 12 h at 37°C and 5% CO₂ to reach a maximal expression of HSP72 (18, 35). AMs were lysed with 100 µl of lysing buffer (6). Samples were denatured by boiling for 15 min and quantified with the MicroProtein Determination kit (Sigma Diagnostics, St. Louis, MO).

One-dimensional Western blot analysis of HSP72. Electrophoresis of 25 µl of AM homogenate (2.5 × 10⁶ AMs) (32, 33) was performed through a 10% SDS-polyacrylamide gel with the Mini-PROTEAN apparatus (Bio-Rad Laboratories, Richmond, CA). Samples were then electrophoretically transferred to nitrocellulose Hybond-C (Amersham, Arlington Heights, IL). Nonspecific sites were blocked overnight with a Tris-buffered saline-Tween solution plus 5% dried milk. The nitrocellulose filter was incubated with a 1:1,000 dilution of the primary antibody [mouse monoclonal antibody specific for the inducible form of HSP70 (HSP72); StressGen Biotechnologies, Vancouver, BC] in the blocking solution for 1 h at room temperature. After this first incubation, the membrane was washed with Tris-buffered saline-Tween. The filter was then incubated for 30 min with a 1:2,500 dilution of the secondary antibody coupled with peroxidase [goat anti-mouse IgG (heavy + light chains)].

The membrane was washed again, and detection was done by chemiluminescence with an enhanced chemiluminescence kit (Amersham).

Human Study

Study population. BAL fluid was obtained from 11 normal subjects (9 men and 2 women) and 18 HP patients (14 men and 4 women). All subjects were nonsmokers and had no unusual environmental exposure except for that responsible for the HP in that group. The mean ages were 27.7 ± 7.6 (SE) yr for the normal subject group and 47.4 ± 14.5 yr for the HP patient group. The diagnosis of HP was based on previously published criteria (29a) and included clinical, functional, radiological, and BAL findings. All HP subjects had an exposure to an at-risk environment (dairy farms, pigeons), clinical symptoms compatible with HP (interstitial lung disease), increased lung function (restrictive pattern with decreased lung diffusion capacity), alveolar infiltrates on high-resolution computed tomograms, and a lymphocytic alveolitis in BAL fluid (>30% lymphocytes).

Heat shock on human AMs. AMs were cultured in 24-wells plates (Becton Dickinson) at ~1 × 10⁶ AMs/well in complete RPMI medium and allowed to adhere for 1 h at 37°C in a 5% CO₂ atmosphere. Nonadherent cells were washed with subsequent Hanks' balanced salt solution. Heat shock was done in a water bath at 39–40°C for 4 h by submerging the plates (37). Control experiments were done at 37°C. At the end of the heat shock, AMs were directly lysed in wells with 100 µl of sample buffer and frozen at −20°C until used. Samples were denatured by a 15-min boiling, and total proteins were quantified with the MicroProtein Determination kit (Sigma).

Fig. 1. Total number of cells and differential counts recovered by bronchoalveolar lavage (BAL) for saline control and Saccharopolyspora rectivirgula (SR)-treated mice. Hatched bars, total cells; cross-hatched bars, macrophages; solid bars, lymphocytes; open bars, neutrophils. Data are means ± SE. For saline group, SE is too small to be visible. *Total number of cells, macrophages, and lymphocytes were higher in SR-treated animals than in saline control animals, P = 0.0001. + Neutrophils were similar in both groups, P = 0.176.

Fig. 2. In vitro expression of heat shock protein (HSP) 72 by alveolar macrophages (AMs) obtained from both groups of mice. Lanes 1 and 2, AMs of saline-treated animals; lanes 3 and 4, AMs from SR-sensitized mice. Nos. at left, temperature in °C. In lanes 2 and 4, SR antigen was added in vitro. After exposure for 20 min at 37°C, no HSP was expressed. Heat shock (42°C for 20 min) resulted in HSP72 expression in all AM cultures. In saline animals, addition of SR further enhanced this expression, whereas antigen failed to do so with AMs from mice who had received this antigen in vivo.
One-dimensional Western blot analysis. The technique used was similar to that used for the animals except that 30 µg of total protein rather than 2.5 x 10^5 AMs were used for loading. Purified HSP72 (StressGen Biotechnologies) was used as a positive control.

Analysis of results. The intensity of the bands obtained was quantified with the National Institutes of Health Image software (available on the Internet at ftp: zippy.nih.gov). Results are expressed as the ratio of the intensity of the signal post-heat shock to that pre-heat shock.

Statistical Analysis

Statistical analyses were done with an analysis of variance (ANOVA) followed by Tukey's honestly significant difference test. When normality and variance assumptions were not respected, an appropriate transformation was done. Differences in HSP72 ratio expression for the HP and control subjects were analyzed by Student's unpaired t-test. All data shown in RESULTS are expressed as means ± SE.

RESULTS

Animal Study

Total and differential counts of cells (in cells/ml x 10^3 BAL fluid) recovered by BAL from the two groups of mice are given in Fig. 1. The antigenic challenge with S. rectivirgula increased the total number of cells from 89.2 ± 7.2 to 1,196.0 ± 176.2 cells/ml x 10^3 BAL fluid, and all cell types were increased after S. rectivirgula treatment (all P < 0.0001).

Figure 2 shows the expression of HSP72 in AMs of mice with and without in vitro stimulation with S. rectivirgula and with and without a heat shock. At 37°C, no HSP expression was seen. Heat shock induced the expression of HSP72 in all AM preparations. For
the animals that received S. rectivirgula in vivo, further stimulation by this antigen in vitro did not enhance the HSP expression.

Human Study

Total and differential cell counts are shown in Fig. 3. HP patients had higher total cell counts than the normal subjects (861.8 ± 78.9 vs. 158.5 ± 13.1 cells/ml × 10^3 BAL fluid, respectively; P < 0.0001). As expected, HP patients had a higher number of lymphocytes than control subjects (516.9 ± 49.9 vs. 18.6 ± 2.6 cells/ml × 10^3 BAL fluid; P < 0.0001). The number of neutrophils was also increased in HP patients.

Because of the larger number of data points for the human studies compared with those for the animal study, we do not present individual blots for each subject. Because the AMs of all human subjects expressed HSP72 at 37°C, we do not present individual blots for each subject. Because there was an age difference between the two groups of subjects, we verified whether this could account for the differences found in this variable. Because the major function of HSPs is to protect cells in periods of stress (4), the blunted ability of AMs to further express HSP72 in response to additional stress in mice with experimental HP and in patients with this disease suggests that this decreased protective mechanism could contribute to the dysfunctions of AMs of HP patients (8). This may indicate that HSP expression reached a maximal level and that the stress response is not further activated by further stress in HP. In fact, the accumulation of stress proteins during chronic conditions of physiological stress can lead to an arrest of biosynthetic events or a reduced ability of affected cells to respond to subsequent exposure to stressful conditions (14). HP recurs with reexposure to the allergen, and a febrile reaction is a classic sign of the disease (7); both are stress conditions for the AMs. These data therefore support the hypothesis that inadequate HSP response to further stress could contribute to the production and the effect of proinflammatory cytokines. It seems obvious, however, that HSPs are not solely responsible for the development of HP. Further studies are required to determine the exact role of these proteins in the modulation of HP and other inflammatory lung diseases.

DISCUSSION

The results of this study show that HSP72 expression by AMs recovered by BAL in response to in vitro stimulation in both the animal model and patients with HP is lower than that of AMs obtained from control animals and normal subjects. This difference was observed by antigenic stimulation of heat-shocked AMs for the mice and by heat shock itself in humans. There was a very wide and unexplained range in the values obtained from the HP patients. We could not find clinical differences in the disease presentation to explain these differences. Also, all subjects from both groups were nonsmokers, and none had been exposed to an unusual environment other than that responsible for their HP. The additional challenge, i.e., by S. rectivirgula, was needed in the mouse study because we could not do a post- to pre-heat shock ratio because of the lack of spontaneous HSP72 expression at 37°C. Further challenge of heat-shocked AMs in the human study would have been interesting and would have allowed a better comparison between the mouse model and the human study. Because of the limited number of BAL cells, this was unfortunately not possible.

Murine AMs did not spontaneously express HSP72 in vitro, whereas AMs obtained by BAL in humans, normal subjects and patients with HP, express this HSP. A possible explanation for this difference between mice and humans is that AMs are stimulated by contaminants in the air we breathe. The mice that we studied were always kept in a controlled nonpolluted environ-

ment. Because there was no correlation between age and HSP response to heat shock, it is unlikely that the age difference between the HP patients and the control subjects could account for the differences found in this variable. Because the major function of HSPs is to protect cells in periods of stress (4), the blunted ability of AMs to further express HSP72 in response to additional stress in mice with experimental HP and in patients with this disease suggests that this decreased protective mechanism could contribute to the dysfunctions of AMs of HP patients (8). This may indicate that HSP expression reached a maximal level and that the stress response is not further activated by further stress in HP. In fact, the accumulation of stress proteins during chronic conditions of physiological stress can lead to an arrest of biosynthetic events or a reduced ability of affected cells to respond to subsequent exposure to stressful conditions (14). HP recurs with reexposure to the allergen, and a febrile reaction is a classic sign of the disease (7); both are stress conditions for the AMs. These data therefore support the hypothesis that inadequate HSP response to further stress could contribute to the production and the effect of proinflammatory cytokines. It seems obvious, however, that HSPs are not solely responsible for the development of HP. Further studies are required to determine the exact role of these proteins in the modulation of HP and other inflammatory lung diseases.

REFERENCES


