Surface Staining and Cytotoxic Activity of Heat-Shock Protein 60 Antibody in Stressed Aortic Endothelial Cells

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Abstract Heat-shock protein (hsp) expression can be induced by high temperature, exposure to cytokines or oxygen radicals, ischemia, hemodynamic overload, or viral infections. To determine whether surface expression of hsp60 occurs in aortic endothelial cells stressed by high temperature or cytokines, cells from rat aortas were cultivated and stained with several types of monoclonal antibodies against hsp60. Other antibodies, eg, those against intercellular adhesion molecule-1 (ICAM-1), or immune response–associated antigens were also used as controls. Positive staining of endothelial cells on the surface and in the cytoplasm was observed after pretreatment of the cells with cytokine-containing medium, tumor necrosis factor-α (TNF-α), or interleukin-1α and labeling with a specific monoclonal antibody against hsp60 (II-13). Fluorescence-activated cell sorter analyses showed that over 80% of living endothelial cells stressed by cytokine-containing medium, by TNF-α, or at 42°C, but not by interleukin-1α, were positively surface stained with this antibody. Increased intensity of immunostaining with antibodies to ICAM-1 and immune response–associated antigen was also seen on the cytokine-stressed endothelial cells. Furthermore, when TNF-α stimulated endothelial cells labeled with 51Cr were incubated with antibody II-13 in the presence of complement, significant lysis occurred. In summary, endothelial cells stressed by high temperature or certain cytokines, eg, TNF-α, express hsp60 in the cytoplasm and on their surfaces, and these cells were susceptible to complement-dependent lysis by hsp60-specific antibody. These observations may be significant for elucidating the mechanisms of the involvement of immune reactions to hsp65/60 in initiating atherosclerosis. (Circ Res. 1994;75:1078-1085.)

Key Words • endothelial cells • heat-shock proteins • surface expression • antibody-complement-mediated cell lysis • atherosclerosis

A previous epidemiological study from our laboratory demonstrated that serum antibodies to mycobacterial stress or heat-shock protein (hsp) 65, an evolutionarily highly conserved molecule produced by almost all cells in response to various forms of stress, were significantly increased in subjects with carotid atherosclerosis compared with those without lesions. This increased antibody level was independent of other established risk factors, such as hyperlipidemia, smoking, hypertension, diabetes mellitus, and obesity. These data provided the first evidence for a strong correlation of hsp65 antibodies with carotid atherosclerosis, suggesting that hsp65 could be involved in the pathogenesis of the disease.

In human atherosclerotic lesions, hsp expression is increased in endothelial cells and macrophages around the necrotic core within the lesions. Homogenates of atherosclerotic lesions when probed by human high-titer sera to mycobacterial hsp65 on Western blots showed a 60-kD protein band. Interestingly, by use of double-immunofluorescence labeling with human anti-hsp65 sera and cell marker–specific antibodies, endothelial cells and macrophages within atherosclerotic lesions were positive, indicating that the serum antibodies against mycobacterial hsp65 cross-react with mammalian hsp60 expressed as an autologous cellular component within the lesions.

In animal experiments, rabbits were immunized with a panel of antigens, including proteins isolated from atherosclerotic lesions and recombinant hsp65 of Mycobacterium tuberculosis. Atherosclerotic lesions developed only in animals immunized with hsp65-containing material, either in the form of mycobacteria or recombinant hsp65 alone. Increased expression of autologous hsp60 and T cells of corresponding specificity have been observed at every stage of atherosclerosis induced by immunization with hsp65 or feeding with a cholesterol-enriched diet. These results suggested that an immune reaction to hsp60 might play an important role in the development of atherosclerosis.

The aim of the present work was to study whether hsp60 is expressed on the surface of arterial endothelial cells and whether hsp60-specific antibodies mediate endothelial cytotoxicity. Hence, we stimulated aortic endothelial cells in vitro with cytokines, examined the expression of hsp60, and determined hsp60 antibody-complement–mediated endothelial cell lysis.

Materials and Methods

Endothelial Cell Culture

Endothelial cells were isolated from aortas of female Lewis rats, weighing between 80 and 120 g (Zentrales Institut für Versuchstierzucht, Hannover, Germany). Rats were killed by
heart puncture under ether anesthesia, and the aorta was removed intact from the heart to the iliac bifurcation, washed with RPMI 1640 medium (Seromed) on ice, and trimmed to remove adventitia with scissors. The aorta was turned inside out with a stainless-steel probe. The aortas with ligated and burned ends were then digested in RPMI 1640 medium containing 1.5 mg/mL collagenase (type IV, No. C5138; Sigma) and 1 mg/mL trypsin inhibitor (No. T6522, Sigma) for 20 minutes at room temperature. The released cells were harvested by rinsing aortas with RPMI 1640 medium supplemented with 20% fetal calf serum (FCS, Boehringer Mannheim), 100 U/mL penicillin, and 100 µg/mL streptomycin and by centrifuging (at 800g) for 5 minutes. The cells were suspended in RPMI 1640 medium containing endothelial cell growth supplement (100 µg/mL, No. Ed760, Sigma) and heparin (100 µg/mL, Sigma), seeded at a concentration of 10⁵ cells per milliliter in a flat-bottomed 96-well plate (Nunc) precoated with 0.1% gelatin (No. G1393, Sigma) in RPMI 1640 medium for 30 minutes at room temperature, and cultured in a 5% CO₂ atmosphere at 37°C. Growth medium was changed every 3 days until the cells had reached confluence (9 to 12 days). For subculture, the cells were incubated in RPMI 1640 medium containing 1 mg/mL trypsin and 0.5 mg/mL EDTA at room temperature for 3 to 5 minutes, the supernatant was discharged, and the cells were resuspended in growth medium at a concentration of 2×10⁵ cells per milliliter and cultivated in 24-well plates or 50-µL flasks (Falcon, Becton Dickinson & Co) precoated with 0.1% gelatin. Cells were identified as endothelial cells by morphology, immunofluorescent staining, and fluorescence-activated cell sorter (FACS) analysis. The fourth to sixth passages of endothelial cells were used for the experiments.

### Immunofluorescence

The sources and specificities of all antibodies used in the present study are summarized in the Table. Characteristics of the anti-hsp60 monoclonal antibodies used in the present study have been described previously⁷⁻⁹ and can be summarized as follows: II-13, a murine monoclonal antibody, developed by immunizing a mouse with human hsp60, recognizes an epitope of amino acid residues 288 to 366 of mammalian hsp60 and does not cross-react with mycobacterial hsp65, hsp70, or other cellular proteins.⁷ The murine monoclonal antibody ML-30 has been found to react with a sequential epitope of mycobacterial hsp65 within residues 311 to 322, of which a tetrapeptide (residues 315 to 318) is shared with the mammalian hsp60 sequence.⁸ Another two murine monoclonal antibodies (LK1 and LK2) recognize mammalian hsp60 amino acid sequence (residues 383 to 447), with (LK1) or without (LK2) cross-reactivity with the bacterial counterpart.⁹ The monoclonal antibody OX2 specifically recognizes a 45-kD glycoprotein on the membrane of rat endothelial cells.¹⁰ Fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse Ig was purchased from Dakopatts (No. F261).

Immunofluorescence studies were performed as previously described,¹¹ with slight modifications. Briefly, endothelial cells growing in eight-well slide chambers (Nunc) were incubated for 12 hours at 37°C with 20% conditioned medium (vol/vol) prepared by cultivating normal rat spleen cells with concanavalin A (Con A, Pharmacia) in serum-free RPMI 1640 medium for 48 hours. The supernatant, termed conditioned medium, was harvested, concentrated, and absorbed with rat red blood cells to remove Con A.¹² After a washing with RPMI 1640 medium, living cells were either treated with monoclonal antibodies for surface staining for 1 hour at room temperature or air-dried for 20 minutes, fixed in acetone for 10 minutes, and then probed by the antibodies for 30 minutes at room temperature. Rabbit anti-mouse Ig-FITC conjugate was used as secondary antibody. Finally, the slides were mounted in gelvatol/PBS (1:10 [vol/vol], Monsanto) and examined in an fluorescence epi-illumination microscope equipped with appropriate filters (Leitz Ortholux II).

### FACS Analysis

Endothelial cells were pretreated at 42°C for 30 minutes, followed by a 3-hour culture at 37°C, or preincubated with the conditioned medium (20% [vol/vol]), tumor necrosis factor (TNF-α [50 U/mL]), or interleukin (IL)-1 (500 U/mL, Boehringer-Institut) in RPMI 1640 medium supplemented with 20% FCS at 37°C for 12 hours. For flow cytometric analysis, endothelial cells dissociated with trypsin-EDTA solution were incubated in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA, Sigma) in a total volume of 100 µL with predetermined, appropriately diluted monoclonal antibodies for 1 hour at 4°C. After three PBS–1% BSA washes, the cells were incubated with the FITC-conjugated Ig rabbit anti-mouse Ig for 30 minutes and washed again. Fluorescence measurements were performed by FACS analysis (FACScan, Becton Dickinson & Co). Details of FACS settings and methods of quantification of fluorescence intensity of labeled cells have been described previously.¹³

### Antibody–Complement–Mediated Cytotoxicity

Confluent endothelial cells were preincubated with or without TNF-α (500 U/mL) for 12 hours, washed with RPMI 1640 medium, and labeled with 5 µCi of ⁵¹Cr in 60 µL of RPMI 1640 medium supplemented with 20% FCS per well at 37°C for 1.5 hours. After three washes with RPMI 1640 medium to remove the ⁵¹Cr excess, monoclonal antibodies against hsp60 (II-13 or ML-30) or anti-actin were added at various concentrations in 100 µL of medium and incubated at 37°C for 7 hours in the presence of guinea pig serum as a source of complement. At the end of the incubation period, 100 µL of cold medium (4°C) was added to each well. The plates were centrifuged at 4°C at 400g for 10 minutes, then 150 µL of the supernatant was removed, and radioactivity was determined in a gamma counter.

### Statistical Analyses

Statistical analyses were performed by paired Student’s t test.

### Results

#### Endothelial Cells

For aortic endothelial cell culture, we developed a method wherein the rat aorta was turned inside out to extensively expose the intimal endothelium to, and hide adventitia from, the enzyme solution during the diges-
Untreated cells properties, including positive staining pretreated with the conditioned medium (2E). When the cell-associated fluorescence intensity fluorescein isothiocyanate-conjugated of the cells in microsopy, nofluorescence pattern apparent growth observations, morphological observations of endothelial cells, staining on medium from (Fig 1) by the endothelial cell-specific monoclonal antibodies against endothelial cells, positively staining medium from (Fig 2F). hsp60 monoclonal antibodies 11-13 and ML-30 (Fig 1) by the endothelial cell-specific monoclonal antibody OX2 (Table). This method, which yields pure aortic endothelial cells, could be useful for investigating the role of the cells in atherogenesis, since endothelium from different blood vessels shows various phenotypic and functional properties, including differential expression of adhesion molecules and some other antigens.14

To determine hsp60 expression by cytokine-stimulated endothelial cells, the aortic cells were treated for 12 hours at 37°C, with or without the conditioned medium from Con A–cultivated normal rat spleen cells, and labeled with hsp60 monoclonal antibodies. Weak staining on the surface of living unstimulated endothelial cells was found with one of the monoclonal antibodies (II-13) against an epitope localized between amino acid residues 288 to 366 of hsp60, whereas strong positive staining was observed on the surface of cells pretreated with the conditioned medium (Fig 2A and 2E). When the same procedure was used to stain the cells with another monoclonal antibody (ML-30) recognizing an epitope spanning amino acid residues 315 to 318 of hsp60, no positive labeling was observed on the surface of the conditioned medium–treated (Fig 2B) or untreated cells (Fig 2F). However, when endothelial cells pretreated with the conditioned medium were air-dried and fixed with acetone, the cells revealed intense intracellular immunostaining with both antibodies II-13 and ML-30 (Fig 2C and 2D, respectively). A similar reaction was found using other fixatives, such as

Fig 1. Fluorescence-activated cell sorter (FACS) analysis of endothelial cells. Endothelial cells, dissociated in trypsin-EDTA solution, were incubated with normal mouse serum (A) or monoclonal antibodies against endothelial cells (OX2, B) for 1 hour at room temperature. After washing with RPMI 1640 medium, the endothelial cells were incubated with anti-mouse Ig fluorescein isothiocyanate-conjugated rabbit Ig for 30 minutes. The cell-associated fluorescence intensity was measured by FACS (Becton Dickinson & Co). Note that cells labeled positively for OX2 constituted 95% of the total population.

Fig 2. Photomicrographs showing detection of heat-shock protein (hsp) 60 in endothelial cells. Endothelial cells were cultured in eight-well slide bottles with (A through D, stressed) or without (E through H, not stressed) 20% conditioned medium (vol/vol) for 12 hours at 37°C. After being washed with RPMI 1640 medium, the living endothelial cells were incubated with anti-hsp60 monoclonal antibodies II-13 (A and E) and ML-30 (B and F) for 1 hour or air-dried for 20 minutes at room temperature, fixed with acetone for 10 minutes, and then incubated with II-13 (C and G) or ML-30 (D and H) for 1 hour. The reaction was visualized by anti-mouse Ig fluorescein isothiocyanate-conjugated rabbit Ig. Original magnification ×250.
ethanol and chloroform (data not shown; see Reference 3). The pattern of hsp60 staining on the cells showed a spot- or string-shaped appearance. Unstressed fixed endothelial control cells remained negative, both with monoclonal antibodies II-13 (Fig 2G) and ML-30 (Fig 2H).

To determine which type of cytokines was able to induce hsp60 surface expression by endothelial cells, purified human recombinant TNF-α, IL-1α, showing activity with rat cells, and the conditioned medium were added to the endothelial cells. FACS analyses of living endothelial cells revealed that >80% of the cells stressed by TNF-α or the conditioned medium were positively stained by anti-hsp60 antibody II-13, whereas the cells preincubated with IL-1α did not show immunostaining (Fig 3A through 3F), further supporting the notion that surface expression of hsp60 occurred on endothelial cells stressed by cytokine(s), ie, TNF-α. Furthermore, LK1 and LK2, recognizing residues 383 to 447 of hsp60, did not label the surface of stressed endothelial cells (data not shown). In addition, the viability of labeled endothelial cells was determined by FACS scatter analysis (always >85%), and only living cells were gated and subjected to immunofluorescence measurements.

To assay the possibility of hsp60 surface staining in response to heat, endothelial cells were stressed by high temperature and labeled with the antibodies. Heat stress is believed to increase the synthesis of hsp and to decrease that of other cellular proteins. Again, strong positive surface staining with the monoclonal antibody II-13 was observed on endothelial cells treated at 42°C for 30 minutes, but no significant reaction appeared with the antibody ML-30 (Fig 3G and 3H).

When endothelial cells were stimulated by conditioned medium, intercellular adhesion molecular 1 (ICAM-1) was also found to be expressed at high levels on the stressed versus unstressed cells, the latter show-
Cells stressed by monoclonal antibody against rat ICAM-1 (Fig 4A and 4B). Simultaneously, positive immunofluorescence staining with a monoclonal antibody to rat immune response-associated (Ia) antigen was seen in the stressed endothelial cells but not in the unstressed cells (Fig 4C and 4D).

When endothelial cells were preincubated with cytokine-containing medium and incubated with monoclonal antibodies against rat ICAM-1, Ia antigen, or CD3, FACS analyses showed that all cells stained positive for ICAM-1, most reacted with anti-Ia, and all were negative for CD3 (Fig 5).

**hsp60 Antibody–Mediated Cytotoxicity**

Interestingly, $^{31}$Cr release by the labeled endothelial cells stressed by TNF-$\alpha$ occurred in the presence of hsp60-specific antibody and complement. Significant $^{51}$Cr release by stressed endothelial cells was only induced by antibody II-13, not by ML-30 or an anti-actin antibody ($P<.01$), which served as a nonrelated control (Fig 6). These data provide further evidence for the availability of at least a certain bona fide or cross-reacting epitope(s) of hsp60 on the surface of stressed endothelial cells.

**Discussion**

Previous studies have shown the presence of antibodies and T lymphocytes specifically reacting to hsp65/60 in peripheral blood of humans and animals with or without clinical manifestations. Possible mechanisms for the induction of specific immune reactions may involve the...
These molecules can be both released from the cell and bound to its surface. It was recently demonstrated that human hsp65 antibodies label the surface of endothelial cells analyzed by confocal laser-scanning fluorescence microscopy and that these antibodies mediate endothelial cytotoxicity via antibody-complement-mediated cell lysis and antibody-dependent cellular cytotoxicity in vitro (G. Schett et al, unpublished data, 1994). This could be also true for the in vivo situation; i.e., hsp65 antibodies could directly bind to surface-expressed hsp60 epitopes by cross-reaction due to the known interspecies amino acid sequence homology. Subsequently, the antigen-antibody complex may activate complement and attract monocytes, entailing injury to endothelial cells. Then, a series of additional events in the arterial intima occur during atherogenesis, such as foam cell formation. Furthermore, once atherosclerotic lesions emerge, T cells, macrophages, and smooth muscle cells within the lesions release a panel of cytokines, including TNF-α, IL-1, and γ-interferon (for review, see Reference 33). These cytokines could further stimulate endothelial cells, thus amplifying mononuclear cell adhesion and recruitment into the intima. Macrophages within the lesions positively stained by human anti-hsp65 antibodies express hsp60 on their surface, which may result in complement-mediated cell lysis that may contribute to the perpetuation of the disease and the formation of the necrotic core in advanced lesions.

A prominent feature of atherosclerosis is the interaction of blood leukocytes with various cellular components of the arterial wall. One of the earliest detectable events is the adherence of mononuclear cells to the endothelial lining of large arteries. Subsequently, monocytes migrate into the intima, where they accumulate and become transformed into lipid-engorged foam cells. In hypercholesterolemic rabbits, aortic endothelial cells have been demonstrated to express vascular cell adhesion molecule-1 (VCAM-1) during atherogenesis. In human atherosclerotic plaques, increased expression of ICAM-1 and other adhesion molecules was recently reported. Adhesion molecules are definitely needed for T-cell attachment to endothelium because of the relatively low binding affinity of the T-cell antigen receptor to its antigen. Our data show that aortic endothelial cells responding to cytokine-containing medium coexpress ICAM-1, IIa antigen, and hsp60 on their surface. Thus, in vivo hsp60-specific T cells could show prolonged adherence to stressed endothelial cells, recognizing adhesion molecules and hsp60 expressed on the surface and thus evoking endothelial injury via cell-mediated cytotoxicity.

In the present study, several monoclonal antibodies against various epitopes of hsp60 were used to stain stressed endothelial cells. Surface staining of endothelial cells was obtained by antibody II-13, recognizing amino acid residues 288 to 366 of hsp60, but not by ML-30, recognizing residues 315 to 318, and LK1 and LK2, recognizing residues 383 to 447. Recently, expression of hsp60 on the surface of human lymphoma cells using the antibody II-13 was demonstrated by a variety of techniques. Physiologically, hsp60 can maintain certain polypeptides in an unfolded state, thus facilitat-
ing their translocation across membranes. In this process, a portion of the hsp60 molecule might extend beyond the cellular plasma membrane to the surface.\textsuperscript{30} hsp70 was also shown in increased levels in human atherosclerotic lesions, although it is not clear whether hsp70 is involved in the pathogenesis of atherosclerosis.\textsuperscript{33} Further studies of this area are now under way in our laboratory. In view of the possible role of hsp65/60 in the pathogenesis of atherosclerosis, the hsp60 domain, recognized by antibody II-13, ie, amino acid residues 288 to 366, should be considered as an appropriate target for further investigation, including possible sequence homologies to any other proteins.

As mentioned above, atherosclerosis-associated cells can secrete various cytokines, eg, IL-1, IL-4, IL-6, IL-8, TNF-\(\alpha\), \(\gamma\)-interferon, and monocyte chemotactic factors.\textsuperscript{33} In the present study, Con A–cultivated spleen leukocyte–conditioned medium was used as a cytokine source to stimulate endothelial cells in vitro, mimicking the in vivo condition, since a variety of cytokines, ie, \(\gamma\)-interferon, TNF, and interleukins, is induced by T cells stimulated by the mitogen Con A.\textsuperscript{42} In addition, although it is known that hsp expression can be induced by TNF\textsuperscript{35} and that adhesion molecules can be activated by IL-1 and TNF (for review see Reference 43), we emphasize a coordinated role of cytokines in endothelial cell stimulation, which may shed more light on the role of cytokines on endothelial pathophysiology in vivo.

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