Fluid Shear Stress Induces Heat Shock Protein 60 Expression in Endothelial Cells In Vitro and In Vivo

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Abstract—Recent investigations indicate that the initial event in the pathogenesis of atherosclerosis involves an (auto)immunologic injury to the vessel wall. Heat shock proteins (hsp), which are expressed on the endothelial cell surface, constitute possible autoantigens. After being exposed to shear stress of 30 dyne/cm² in vitro by means of a rotational viscometer, human umbilical vein endothelial cells were immunohistochemically stained for hsp 60 by the monoclonal antibody ML-30; static control cells were negative. Maximal hsp 60 induction was observed after 12 hours of hemodynamic stress. In Northern blots, the level of hsp 60 mRNA was markedly increased after only 1 hour of shear stress in human umbilical vein endothelial cells compared with static control cells. In vivo investigations in Lewis rats confirmed these in vitro findings: the intima and media of frozen sections of the right common carotid artery exposed to increased wall shear stress (after ligation of the left common carotid artery) were stained for hsp 60. The vessel wall of the left low-shear-stress–exposed side was negative. These findings demonstrate that shear stress results in hsp 60 induction in endothelial cells in vivo and, in vitro, providing the prerequisite for humoral and cellular reactions to endothelial hsp in the earliest stages of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2000;20:617-623.)

Key Words: atherosclerosis ■ endothelial cells ■ endothelium ■ shear stress ■ heat shock protein

It is well known that cells respond to a variety of environmental stresses (eg, elevated temperature, heavy metals, toxins, and free radicals) by rapid production of a family of proteins called the heat shock proteins (hsp).1–3 This response is markedly conserved throughout evolution, from bacteria to humans. Hsps are divided into 4 major families according to their molecular mass (30, 60, 70, and 90 kDa). Some of them (60-kDa and 70-kDa families) function as “molecular chaperones.” They bind to immature or partially denatured proteins, helping them to fold into their native conformation and regain their functional activity.

Some microbial hsps are important target antigens, eliciting protective immune responses against many infectious organisms. As a side effect, hsps evoke interest in pathology as autoantigens by “antigenic mimicry.” The have been associated with diseases like rheumatoid arthritis,4 diabetes mellitus,5 systemic sclerosis,6 rejection of transplanted organs,7 and atherosclerosis.8–10 A previous epidemiological study11 from our laboratory had demonstrated that serum antibodies to mycobacterial hsp 65, which is a homologue of human hsp 60, were significantly increased in clinically healthy subjects with sonographically demonstrable 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. Serum concentrations of hsp 65 antibodies were also elevated in patients with coronary heart disease compared with healthy controls.12 Furthermore, in atherosclerotic lesions of rabbits and humans, endothelial cells showed higher levels of hsp 60 than those in other parts of the normal arterial intima.13,14 In fact, immunization of rabbits with recombinant mycobacterial hsp 65 induced the development of atherosclerotic lesions at characteristic predilection sites, such as the curve, bifurcation, branch, or attachment of arteries.15

The flow of blood is associated with shear stress, the reactive force per unit area, which acts in the direction of blood flow on the surface of the inner wall of the blood vessel. In the simplifying case of laminar Poiseuille-Hagen flow, wall shear stress is proportional to flow velocity and medium viscosity and is inversely proportional to the third power of the internal radius.16 Although the effect of inflammatory mediators and cytokines on endothelial cells has been extensively studied, less is known about the effect of shear stress. Endothelial cells rapidly respond to a change of shear stress by signal transduction events, such as an increase of intracellular calcium,17–19 decrease of intracellular pH,20 or increase of inositol trisphosphate and diacylglycerol.21,22 The expression of many genes is altered by shear stress. Examples of such genes are c-fos23–25 platelet-derived growth factor B
chain, mitogen-activated protein kinases (ERK/INK), and intercellular adhesion molecule-1.

Atherosclerosis is restricted to arteries, and venosclerosis usually does not exist. However, if a vein is transferred to a site subjected to arterial blood pressure, as in coronary bypass surgery, sclerosis and stenosis may often be observed. The patency rate of aortocoronary saphenous vein bypass grafts is estimated at 66% in 10 years. The histopathologic examination of surgically removed saphenous vein bypass grafts showed that stenosis resulted either from intimal fibrocellular proliferation or mainly from progressive venosclerosis.

We speculate that increased hemodynamic stress may be the decisive factor for the occurrence of venosclerosis when veins are exposed to arterial pressure. However, shear forces should not be regarded apart from other factors (eg, oxidized LDL, cytokines, and elevated blood glucose) to which vascular endothelium is exposed. Possibly, prestress by arterial blood pressure lowers the threshold for hsp 60/adhesion molecule induction by other stress factors, including classic risk factors for atherosclerosis. To establish the missing link between shear forces and the immunologic hypothesis of atherosclerosis, we addressed whether hemodynamic forces could also induce hsp 60 expression in endothelial cells in vitro and in vivo.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cords obtained from the Department of Gynecology and Obstetrics of the Innsbruck University Hospital by collagenase type II (Sigma Chemical Co) digestion for 15 minutes. Subsequent cultivation was maintained at 37°C in a humidified atmosphere (5% CO₂ in air) by using endothelial cell growth medium (Promocell, catalogue No. C-22010). Petri dishes with a diameter of 100 mm (Falcon, item 3003, Becton Dickinson Co) were precoated with 28 μg/mL human fibronectin (HFN, Inotech AG). Cells were identified as endothelial by the typical cobblestone-like morphological appearance and positive von Willebrand factor immunohistochemical staining (mouse/anti-human von Willebrand factor antibody, Dako, code No. M0616). Only cultures of high purity with fibroblast contamination <3% were accepted for further experimentation. Trypsin/EDTA solution (0.025%/0.02%, Sigma) was applied for subcultivation. Second and third passages were used for shear stress experiments and static controls. Endothelial cells were seeded into precoated Petri dishes (Falcon, item 3003) at seeding densities of 0.8 to 1.2×10⁶ cells per dish. Experiments were initiated after a cultivation period of 5 days, when a semiconfluent monolayer had been established.

Shear Stress Device

A semiconfluent monolayer of HUVECs was fed with growth medium (see above) 24 hours before the onset of hemodynamic stress. The monolayer was then exposed to laminar shear stress by use of a purpose-built cone and plate viscometer, specifically designed to accept 100-mm-diameter culture plates at 37°C and a humidified atmosphere of 5% CO₂. Reynolds number, R, and fluid shear stress magnitude, τ, were computed at different values of radius, r, as shown in Equations 1 and 2 below according to Sdoggos et al:

\[ R = \frac{r^2 \omega \alpha^2}{12 \nu} \]

\[ \tau = \frac{\mu \omega}{\alpha} \left[ 1 + 2.58 \left( \frac{R^{5/2}}{3.5 + R} \right) - 0.86 \left( \frac{R^{5/2}}{(3.5 + R)^2} \right) \right] \]

In this equation, μ is viscosity, ν is kinematic viscosity, ω is rotational velocity, and α is the cone angle. Static viscosity of the medium, obtained by measuring kinematic viscosity with a calibrated capillary viscometer and multiplying by the density of the medium, was 0.79 centipoise at 37°C. Average Reynolds number and shear stress magnitudes were obtained by integrating their respective values over the area of the tissue culture plate. Our cone with an angle of 0.5° and spinning at 5 revolutions per second achieved an average shear stress of 30 dyne/cm² and Reynolds number of 0.20. Other shear stress magnitudes were attained by variation of rotational speed.

Immunohistochemistry

Sections were fixed in absolute ethanol for 10 minutes at room temperature. Subsequently, the sections were placed in a humidified chamber and overlaid with the monoclonal antibody ML-30 (gift from Dr J. Ivanyi, London, UK) recognizing a cross-reactive epitope of mycobacterial hsp 65 and human hsp 60. After they were washed with Tris buffer (0.005 mol/L, pH 7.6), the sections were incubated with rabbit anti-mouse immunoglobulin antibodies (Dako Corp) for 30 minutes. After further washes in Tris buffer, the sections were incubated with alkaline phosphatase mouse anti–alkaline phosphatase complex (Dako) for 30 minutes, washed in Tris buffer 3 times, and developed for 10 minutes at room temperature with use of a substrate solution containing 9.8 mL Tris buffer (0.1 mol/L, pH 7.8), 0.2 mL dimethylformamide, 8 mg naphthol AS-MX phosphate, 3 mg levamisole, and 10 mg Fast Red TR salt (Sigma).

RNA Isolation and Hybridization

The acid guanidinium thiocyanate–phenol–chloroform method was used to isolate total cellular RNA. After determination of RNA purity and concentration, 10 μg of total RNA was fractionated with the use of 1% agarose gels containing 8% formaldehyde and 0.04 mol/L Bicin (pH 8.4, Serva). RNA was then transferred overnight by capillary action in 10× SSC (3 mol/L sodium chloride and 300 mM/L sodium citrate, pH 7) on a blotting membrane (Zeta-Probe, Bio-Rad) and immobilized by UV irradiation. Prehybridization and hybridization of membranes were accomplished in Church buffer (1% BSA, 7% SDS, 0.075% EDTA, and 0.5 mol/L sodium phosphate). Northern blot hybridization was performed with a random-primer ³²P-labeled fragment of hsp 60. After incubation, the blots were washed in 2× SSC/1% SDS for 15 minutes at room temperature, followed by 2 washes of 20 minutes in 05× SSC/1% SDS at 65°C and then exposed to x-ray film.

In Vivo Experiments

Adult female Lewis rats (Harlan-Winkelmann, Borchen, Germany), weighing 200 to 300 g, were anesthetized with an intraperitoneal injection of 5 mg Vetarnacol (Veterinaria AG). A cervical midline incision was made caudal to the thyroid cartilage, and then the left common carotid artery was exposed and ligated just rostral to the origin of the superior thyroid artery branch with 2.0 silk. The incision was closed in layers, and the animal was allowed to recover. According to Walpola and colleagues, this procedure results in an increase of shear stress in the right common carotid artery of ~170% and a decrease in the downstream left carotid artery of 70%.

Results

In Vitro Data: Hsp 60 Staining

At least 4 different HUVEC isolates were used for the in vitro studies. Semiconfluent monolayers of HUVECs underwent laminar shear stress of 30 dyne/cm² in a cone-plate viscometer for 3, 6, 12, and 24 hours. Fluid shear stress induced a characteristic cell-shape change and cell alignment, as previously described. Cells appeared longer and narrower and displayed a protrusion of the nuclear area. After 24 hours of constant shear stress, there was complete and uniform alignment in the direction of the flow.

HUVECs were immunohistochemically stained with the monoclonal antibody anti–hsp 65/60 ML-30. Static controls showed very weak, if any, hsp 60 staining, whereas mechan-
ically stressed HUVECs revealed distinctive positive staining (Figure 1). Increased expression of hsp 60 was found at 6 hours, and there was a peak at 12 hours and a subsequent slight decline at 24 hours. At 12 hours, the percentage of responsive cells was determined as 94% (average of cell counts from 3 subsequent experiments). Positive controls were obtained by means of a heat shock of 42°C for 30 minutes. Figure 2 demonstrates a positive “dose”-response correlation between the level of shear stress and immunohistochemical staining for hsp 60. Semiconfluent HUVECs underwent 6 hours of laminar shear stress of varied intensities (5, 20, and 50 dyne/cm²). Shear forces corresponding to venous flow (5 dyne/cm²) only had a minimal effect. However, simulating arterial conditions in vitro (20 dyne/cm²) produced more distinctive, but moderate, staining. Excessive shear forces >50 dyne/cm², as found physiologically at the branching of arterial vessels, induced strongly positive staining. Figure 3 illustrates how long an episode of elevated shear stress must persist to induce a significantly increased expression of hsp 60. HUVECs underwent 5, 10, or 30 minutes of shear stress (30 dyne/cm²) and remained in the incubator under static conditions until 6 hours after the onset of shear forces. Five or 10 minutes of increased hemodynamic stress did not significantly influence hsp 60 protein levels, as determined by immunohistochimetry, whereas 30 minutes of shear stress was enough to cause a translational upregulation of hsp 60.

**Hsp 60 mRNA**

Semiconfluent monolayers of HUVECs were subjected to laminar shear stress of 30 dyne/cm² in a cone-plate viscometer for 30 minutes (plus 30-minute static), 1 hour, 3 hours (plus 3-hour static), and 8 hours. mRNA was isolated from these cells at the above time points after onset of shear stress. The cells expressed hsp 60 transcripts after 30 minutes of shear stress and 30 minutes of relaxation at significantly higher levels than did static controls (Figure 4A). However, as expected and known from previous studies, static controls were not completely negative for hsp 60 mRNA but showed a baseline expression of hsp 60 mRNA, which was possibly due to the stress of cell culture itself. Hsp 60 mRNA levels increased between 1 hour and 8 hours of constant shear stress. Cells in lane C underwent 3 hours of shear stress and were allowed to rest for a further 3 hours before harvesting of the cells. During the 3 hours of relaxation, hsp 60 mRNA significantly decreased. Figure 4B demonstrates a correlation between dose of shear stress and hsp 60 mRNA formation, as also shown above on the protein level.
In Vivo Data: Hsp 60 Staining
Three animals per time point were euthanized 3, 6, 12, and 24 hours after ligation of the left common carotid artery. Left and right common carotid artery samples were put into slices of liver tissue for processing purposes and frozen in liquid nitrogen. Serial cross sections were immunohistochemically stained with monoclonal antibodies against mycobacterial hsp 65 (ML-30). Sham-operated rats and rats that were anesthetized only (without subsequent operation) showed no staining on either side. Intimal endothelium, media, and adventitia of left carotid arteries, experiencing low shear stress, showed weak, if any, hsp 60 staining at all time points. Carotid endothelium and media from the right side exposed to high shear stress revealed distinctive positive staining. The time course is shown in Figure 5: hsp 60 staining of intima and media started between 3 and 6 hours after the operation and steadily increased from 12 to 24 hours.

Discussion
Previous work 8–11,13–15 defined a possible role of hsp 60 as an (auto)antigen in atherogenesis. Because hsp 60 is imperceptibly expressed constitutively on the cell surface, a certain prestress is a prerequisite for making endothelial cells immunologic targets of anti–hsp 60 antibodies and/or effective T cells. Using a combination of immunohistochemistry and Northern analysis, we were able to demonstrate that fluctuations in blood flow can exert such a prestress on endothelial cells, upregulating hsp 60 expression in vitro and in vivo. Although the in vitro fluorescent staining of hsp 60 is clearly intracellular in Figures 1 to 3, there is ample evidence from previous work that hsp 60 is also expressed on the surface of stressed endothelial cells.13 As a matter of fact, it has also been shown that anti–hsp 60/65 antibodies are able to lyse stressed but not unstressed endothelial cells.39

The in vivo studies (Figure 5) demonstrate that staining of hsp 60 is not restricted to the intima but also includes the media and adventitia. This finding complies with the previous observation that hypertensive stress in vivo lead to the expression of hsp 70 by vascular smooth muscle cells,40 and we deem it likely that similar mechanisms may underlie the expression of hsp 60 in our experiments at the mentioned sites. As a matter of fact, it has been demonstrated previously that smooth muscle cells in atherosclerotic lesions express hsp 60.14

Static controls did not stain for hsp 60 protein, but we constantly observed a basal hsp 60 mRNA transcription of static HUVECs in vitro. Although culture medium was exchanged every 48 hours and pH was kept stable, the basal hsp 60 transcription is possibly due to the stress of cell culture itself. Another discrepancy between Northern analysis and fluorescent staining is the fact that in Figure 4B a lack of hsp 60 mRNA induction with 20 dyne/cm² is noted, whereas in Figure 2, positive immunohistochemical staining for hsp 60 is seen with only 5 dyne/cm². However, we do not feel that this is an experimental error, because both phenomena were very reproducible.

To our knowledge, the only other stress protein that has been studied regarding the application of shear stress on endothelial cells is the small molecular mass hsp of 27 kDa (hsp 27).41 Interestingly, after an application of shear stress of 16 dyne/cm², hsp 27 became more highly phosphorylated, whereas hsp 27 antigen levels did not change. Thus, the modulation of hsp 27 by shear stress is merely posttranslational.
Shear stress is a less ideal parameter for predilection sites of atherosclerosis. Gibson et al\textsuperscript{42} used quantitative angiography to calculate coronary arterial diameter during a 3-year period in 20 arterial coronary segments. Low shear stress was correlated with an increased rate of atherosclerosis progression. However, the emphasis on low shear stress as a dominant factor in the predilection for localization of atherosclerotic lesions is not justified in view of the development of atherosclerosis at sites of high shear stress. In the present study, we selected higher shear stress compared with physiological arterial flow. For in vitro tests, 30 dyne/cm\textsuperscript{2} was used, and the wall shear stress in the right common carotid artery increased from \( \approx 12 \) dyne/cm\textsuperscript{2} to \( \approx 30 \) dyne/cm\textsuperscript{2} after ligating the left common carotid artery in vivo.

Figure 3. Minimal duration of an elevated shear stress (ss) episode for hsp 60 induction. HUVECs underwent 5, 10, or 30 minutes of ss (30 dyne/cm\textsuperscript{2}) and remained in the incubator under static conditions until 6 hours after the onset of shear forces. Cells were fixed in absolute ethanol and labeled with monoclonal antibody ML-30 against hsp 60.

Figure 4. A, Hsp 60 mRNA in vitro. Semiconfluent monolayers of HUVECs underwent laminar shear stress of 30 dyne/cm\textsuperscript{2} in a cone-plate viscometer for 30 minutes (plus 30-minute static), 1 hour, 3 hours (plus 3-hour static), and 8 hours. mRNA was isolated from these cells at the above times after onset of shear stress and analyzed by Northern blotting. B, Dose-response experiment between hsp 60 mRNA and shear stress in vitro. Semiconfluent monolayers of HUVECs underwent laminar shear stress of 5, 20, or 50 dyne/cm\textsuperscript{2} in a cone-plate viscometer for 6 hours.

Texon\textsuperscript{43} identified the sites of predilection for atherosclerosis as precisely those locations characterized by a relative reduction in lateral pressure. The lateral pressure is predictable in a nonviscid fluid on the basis of Bernoulli’s theorem, which states that the sum of pressure and the square of the velocity times density divided by 2 is constant for any 2 points of flow on the same stream line. For example, fluid in a vessel with converging boundaries causes the lateral pressure to be reduced at the narrow portion, where the velocity is increased.

The transductional events and transcriptional factors for shear stress–mediated events are currently under investigation in many laboratories. Resnick and colleagues\textsuperscript{44,45} defined a “shear stress response element” in the promoter of the platelet-derived growth factor B chain gene that interacts with DNA binding proteins in the nuclei of shear-stressed endothelial cells to upregulate transcriptional activity.

The transductional events after the onset of shear stress, which finally lead to hsp 60 expression, are not yet clear. One possible mechanism may involve the endothelial nitric oxide synthase (eNOS), which is expressed constitutively in endothelial cells. Shear stress is a powerful modulator of eNOS expression, increasing eNOS mRNA significantly.\textsuperscript{46} Early data from our laboratory show that intracellular nitric oxide is able to upregulate hsp 60 mRNA formation.\textsuperscript{47} Another early event leading to upregulation of hsp 60 mRNA is the
generation of heat shock factor, which also has been shown to be shear stress inducible.47

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References


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