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Expression of Heat Shock Proteins and Stress Fiber Formation within the Arterial Vascular Tree of Adult Rats under Normal Physiological Conditions

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Abstract

In this study, we investigated heat shock protein (HSP) expression and stress fiber (SF) formation in endothelial cells (ECs) within the arterial vascular tree of adult rats under normal physiological conditions. Using quantitative immunofluorescence microscopy, we found no significant differences in expression of HSPs 25, 60, 70, and 90 among ECs in the straight portions of rat arteries. In these regions, ECs appeared spindle-shaped and contained short bundles of central SFs. In contrast, ECs in the curved portions or the branch sites of the arteries, exhibited striking differences in HSP expression. ECs with higher HSP expression were localized at the lesser curvature in the curved portions or the distal site of the branch ostia. Moreover, the ECs became polygonal and contained irregular central SFs at the lesser curvature. At the branch sites, downstream ECs became spear-shaped and contained long, thick bundles of central SFs. Curved portions or branch sites are the regions of disturbed flow at which early atherosclerotic lesions are often found. Our results demonstrate these positional differences in HSP expression associated with changes in SF formation within the arterial vascular tree under non-pathological conditions. Our study provides basic information for understanding stress responses via HSP expression and SF formation in vascular ECs and the pathogenesis of atherosclerotic disease.
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Key words: artery, endothelial cell, heat shock protein, immunohistochemistry, stress fiber

Introduction

Heat-shock proteins (HSPs) are a family of proteins that are induced by heat or other stresses; they are present in the cells of all organisms[1–3]. The ubiquitous nature of the heat-shock response and its phylogenetic conservation suggest that HSPs are essential for cell survival[4]. HSPs are divided into four major families according to their molecular weight;

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these families include the 90-kDa (HSP 90), 70-kDa (HSP 70), and 60-kDa (HSP 60) families and the small HSP family, which contains HSPs of 15 to 42 kDa. Some HSPs serve as molecular chaperones that interact with other cellular proteins to assist in their assembly, disassembly, stabilization, or transport[5,6].

The vascular endothelial cells (ECs) that cover the luminal surface of blood vessels are constantly exposed to physiological stresses of the circulation, such as fluid shear stress, circumferential distention, and hydraulic (blood) pressure[7]. Shear stress on ECs, which arises from the tangential force on the luminal vessel surface, induces them to express HSPs[6,8] and also causes morphological changes, such as stress fiber (SF) rearrangement[9–12]. There is likely a relationship between HSPs and morphological events in ECs exposed to shear stress. Azuma et al.[13] reported that HSP 25/27 may be involved in the regulation of SF formation in ECs exposed to shear stress. Moreover, we have recently shown using an *ex vivo* system of arterial EC sheets that HSP 70 mediates stretch-stress-induced SF formation[14]. In addition, HSPs are believed to play important roles in the pathogenesis of atherosclerosis and in the pathophysiology of cardiovascular disease[15].

Many of these previous studies of HSP or SF or both were performed under non-physiological experimental conditions, i.e., pathological conditions; for example, specialized shear stress devices were used *in vitro* or vessels were ligated *in vivo*. However, little information is available on the site-specific HSP expression patterns and SF formation in ECs within a vascular tree under normal physiological conditions. In the present study, we investigated HSP expression and SF formation in ECs within the arterial vascular tree of adult rats *in vivo*, focusing on the curved or branched arterial regions. Using quantitative immunofluorescence (IF) microscopy of EC sheets, we obtained data that offers basic information for understanding stress responses via HSP expression and SF formation in vascular ECs and the pathogenesis of atherosclerotic vascular disease.

Materials and Methods

Animals

Procedures involving animals and their care were performed according to the Guide for the Care and Use of Laboratory Animals of Nippon Medical School. Virgin Wistar-Imamichi rats aged 9 to 12 weeks were purchased from the Institute for Animal Reproduction (Ibaragi, Japan). The rats were housed and bred with a 12-hour/12-hour light-dark cycle and were given food and water *ad libitum*. Eighty rats were examined.

Preparation and IF Microcopy of EC Sheets

To examine HSP expression in arterial ECs *in vivo*, rats were anesthetized with sodium pentobarbital (Abbott Laboratories, Abbott Park, IL, USA) and fixed by perfusion with 3% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.4. Curved sites or branch ostia of the following arteries were excised (**Fig. 1a–c**): the external carotid artery curvature, inner (zone A) and outer (zone B) luminal walls (**Fig. 1a**); the aortic arch, inner (zone C) and outer (zone D) luminal walls (**Fig. 1b**); the aortic branch site of the brachiocephalic artery, proximal (E zone) and distal (F zone) portions of the flow divider (**Fig. 1b**); and the abdominal aorta branch site of the celiac artery, proximal (zone G) and distal (zone H) portions of the flow divider (**Fig. 1c**). The following straight arterial portions away from curved sites or branch ostia were also excised: the proximal part of the thoracic aorta, the middle parts of the abdominal aorta, the common iliac artery, and the common carotid artery. The excised vessel segments were cut open lengthwise and then immersed in the same fixative for an additional 15 hours at 4℃. After the specimens were washed well in cold 0.01 M phosphate-buffered saline (PBS), they were extracted with acetone for 20 minutes at 4℃ to allow permeation of antibodies into the cells. They were then incubated with 3% skim milk for 1 hour at 4℃ to block non-specific immunoreactions. Subsequently, the specimens were incubated with either rabbit polyclonal anti-mouse HSP 25 antibody (1 : 500 dilution; Stress Gen Biotechnologies, Victoria,

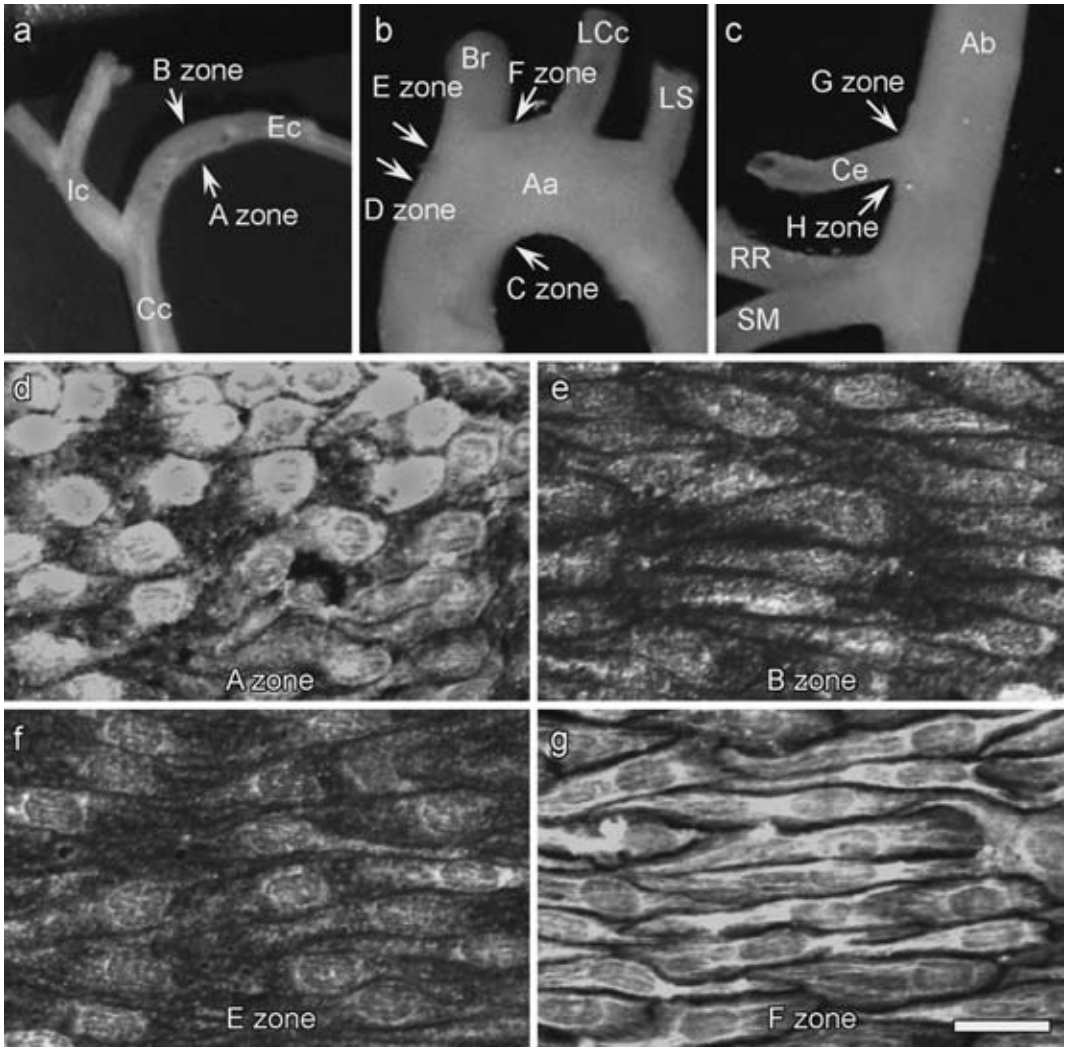

Fig. 1 **a–c** Macroscopic photographs of the curved portions and branch sites of rat arteries. Ab: Abdominal aorta, Br: brachiocephalic artery, Cc: common carotid artery, Ce: celiac artery, Ec: external carotid artery, Ic: internal carotid artery, Aa: aortic arch, LCc: left common carotid artery, LS: left subclavian artery, RR: right renal artery, SM: superior mesenteric artery. The zones examined in the present study are indicated with arrows. **d–g** IF images of HSP 25 expression in EC sheets in zones A **d**, B **e**, E **f**, and F **g** shown in **a** and **b**. Direction of blood flow is from left to right. Scale bar=20 μm.

BC, Canada), mouse monoclonal anti-human HSP 60 antibody (clone LK-1, 5 μg/mL; StressGen Biotechnologies), mouse monoclonal anti-human HSP 70 antibody (clone C92F3A-5, 5 μg/mL; Medical Biological Laboratory, Nagoya, Japan), or mouse monoclonal anti-achlya ambisexualis HSP 90 antibody (clone AC88, 4 μg/mL; Medical Biological Laboratory) for 15 hours at 4℃. According to the manufacturer's technical specifications, the antibodies against HSP 25, HSP 60, HSP 70, and HSP 90 can cross-react with rat HSP 25, 60, 70, and 90, respectively. The specimens were then incubated with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) for 6 hours at 22℃. In control samples, incubations were performed without the primary antibody or with non-immune serum instead of the primary antibody.

EC sheets were prepared as described previously[16]. Briefly, immunostained specimens were pinned flat onto silicone rubber plates with tiny steel pins (Iken Kogyo, Kanagawa, Japan) and then immersed in 95% ethanol for 15 hours at 4℃. After ethanol fixation, as much as possible of the tunica externa and media was removed using fine-tipped

forceps. Arterial EC sheets were then transferred onto slides coated with 4% gelatin, with the endothelial side facing downward.

IF images of the EC sheets were collected with an fluorescence microscope (BX60F5, Olympus, Tokyo, Japan) and captured with an image processor (ARGUS-20, Hamamatsu Photonics, Hamamatsu, Japan). The fluorescence intensity in a given area was measured with image-editing software (Adobe Photoshop version 6.0J, Adobe, San Jose, CA, USA) and used to represent the level of HSP expression. Statistical analysis was performed using analysis of variance software (Stat View version 4.02J, Hulinks, Tokyo, Japan); differences were considered statistically significant at $p<0.05$.

Rhodamine-phalloidin Staining

To visualize actin SFs, rhodamine-phalloidin staining of arterial ECs was performed as described previously[17]. Briefly, rats were first fixed by perfusion with 3% paraformaldehyde solution. The vessel segments of interest were then excised and immersed in 95% ethanol for 15 hours at 4℃. EC sheets were prepared as described above, re-fixed with 3% paraformaldehyde for 15 minutes at 4℃, and then stained with 0.16 μM rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) for 20 minutes at 22℃.

Silver Staining and Determination of Shape Index

To identify cell boundaries, some EC sheets were silver-stained according to the method of Zand et al.[18], and images were captured with the ARGUS-20 image processor. For each vessel segment, 30 to 50 ECs were randomly chosen from the sheets. NIH Image software (version 1.6) was then used to determine the surface area (A) and perimeter (P) of each EC according to the method described by Nerem et al.[19]. The shape index (SI) was obtained using the equation: $SI=4\pi \cdot A/P^2$, where π=the circular constant and is equal to 1 for a circle and 0 for a straight line. Differences in SIs were assessed by the paired *t*-test and were considered significant at $p<0.05$.

Results

Differential Expression of Endothelial HSPs within the Rat Arterial Vascular Tree

Normal physiological expression levels of HSPs 25, 60, 70, and 90 were examined in ECs localized at straight arterial portions away from curved sites or branch ostia. All HSPs examined were detected in ECs in the straight arterial portions. Mean HSP fluorescence intensities are shown in **Figure 2**. No significant differences in HSP fluorescence intensity were evident among the ECs in each straight portion tested (data not shown) or among the straight portions from the various anatomical sources examined (data not shown).

Next, normal physiological HSP expression was investigated in ECs localized at curved sites or branch ostia of rat arteries. In the curved sites tested, HSP 25 was more highly expressed in ECs of the inner luminal walls than in those in the outer luminal walls (**Fig. 2a**, zones A–D). At branch sites, the level of HSP 25 expression was also higher in the distal zones than in the proximal zones (**Fig. 2a**, zones E–H). HSP 60 and 70 exhibited a similar expression pattern to that of HSP 25 in the ECs of the curved or branch sites tested (**Fig. 2b, c**). Endothelial HSP 90 also exhibited differential expression between the proximal and distal zones at the branch sites (**Fig. 2d**, zones E–H) but not between the inner and outer luminal walls at the curved sites (**Fig. 2d**, zones A–D).

SI and SF Distribution in ECs

Silver-stained arterial EC sheets are shown in **Figure 3**, and the derived SI values are shown in **Table 1**. The silver-enhanced cell boundaries confirmed the integrity of the EC monolayers, since silver deposited on the basal lamina of ECs. ECs localized at the outer luminal walls of the curved arterial portions appeared spindle-shaped (**Fig. 3b**), as did those of the straight portions of the same vessels (data not shown). In the outer luminal walls, the endothelial SI was about 0.4 (**Table 1**). In ECs at the outer luminal walls, short bundles of central SFs and continuous circumferential SFs were readily

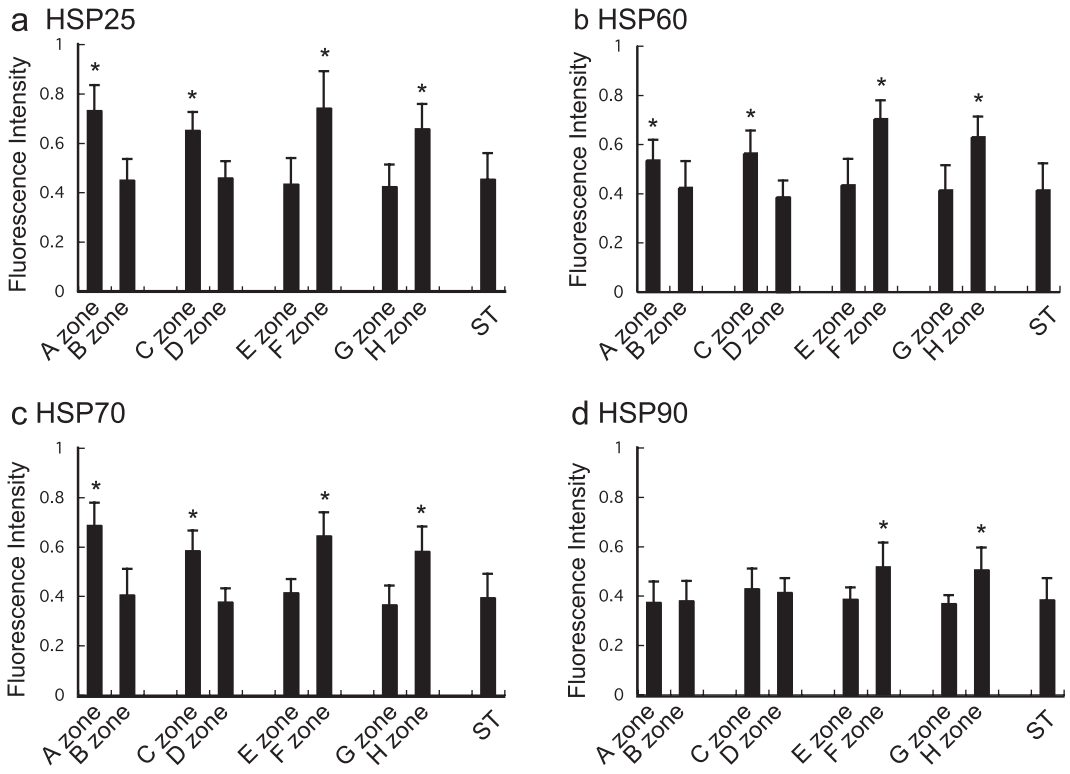

Fig. 2 Quantitative analysis of expression of HSP 25, 60, 70, and 90 in ECs within the arterial vascular tree of adult rats under normal physiological conditions. Values shown are means ± SD. **a:** Endothelial expression of HSP 25 in zones A–H shown in **Figure 1a–c**. The mean fluorescence intensities for endothelial HSP 25 in the straight portions of the abdominal aorta, the common carotid artery, the common iliac artery, and the proximal part of the thoracic aorta are also shown (ST). *Differences between zones A and B, C and D, E and F, and G and H were significant ($p<0.05$). **b:** Endothelial expression of HSP 60 in zones A–H shown in **Figure 1a–c**. The mean fluorescence intensities for endothelial HSP 60 in the straight portions tested are also shown (ST). *Differences between zones A and B, C and D, E and F, and G and H were significant ($p<0.05$). **c:** Endothelial expression of HSP 70 in zones A–H shown in **Figure 1a–c**. The mean fluorescence intensities for endothelial HSP 70 in the straight portions tested are also shown (ST). *Differences between zones A and B, C and D, E and F, and G and H are significant ($p<0.05$). **d:** Endothelial expression of HSP 90 in zones A–H shown in **Figure 1a–c**. The mean fluorescence intensities for endothelial HSP 90 in the straight portions examined are also shown (ST). *Differences between zones E and F and zones G and H were significant ($p<0.05$).

visible **(Fig. 4b)**. In contrast, at the inner walls of the curved portions of the arteries, ECs became polygonal **(Fig. 3a)**, and the SIs of the ECs increased significantly. Although marginal SFs were present in ECs at the inner walls, the central SFs were irregularly distributed **(Fig. 4a)**.

ECs immediately downstream of the rat arterial branch sites were spear-shaped **(Fig. 3d)**, as compared to upstream ECs **(Fig. 3c)**. The SIs of the downstream ECs were significantly smaller than those of the upstream ECs **(Table 1)**. In the downstream ECs, long, thick bundles of central SFs oriented with the blood flow were observed **(Fig. 4 d)**; moreover, the continuous circumferential pattern of SF bundles observed in the upstream ECs **(Fig. 4 c)** and in the ECs of the straight portions (data not shown) was absent.

Discussion

In the present work, we have demonstrated the anatomically regional heterogeneity of HSP expression and actin SF formation within the vascular tree of the rat artery under normal

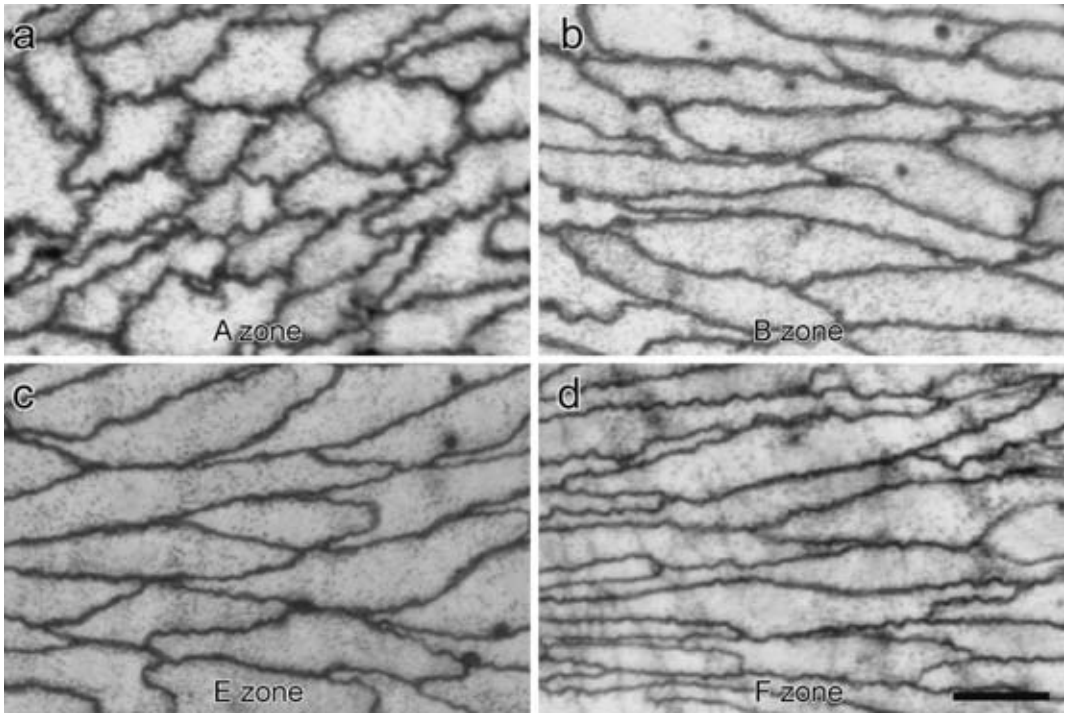

Fig. 3 Silver staining of EC boundaries in zones A **a**, B **b**, E **c**, and F **d** shown in **Figure 1a–c**. Direction of blood flow is from left to right. Scale bars=20 μm.

Table 1 SI[a] values for rat arterial ECs *in vivo*

	External Carotid Artery		Aortic Arch				Abdominal Aorta	
Zone	A	B	C	D	E	F	G	H
SI (mean ± SD)	0.57 ± 0.12*	0.37 ± 0.07	0.53 ± 0.11*	0.40 ± 0.09	0.42 ± 0.08*	0.22 ± 0.06	0.36 ± 0.10*	0.24 ± 0.08
EC-appearance								

[a] SI=4π・A/P^2, where π=he circular constant, which is equal to 1 for a circle and 0 for a straight line.
*Differences between zones A and B, C and D, E and F, and G and H were significant (p<0.05).

physiological conditions. Arterial ECs are continuously exposed to mechanical stresses due to fluid flow even under non-pathological conditions. Findings from previous *in vivo* and *in vitro* studies also indicate that the shape and cytoskeletal structure of vascular ECs reflect environmental differences in blood flow conditions[10–12,20]. In this study, striking differences were observed both in HSP expression and in SF formation among ECs from the curved portions and the branch sites of rat arteries under normal conditions. It is likely that laminar flow disturbances have a great influence on the stress responses of ECs in the curved portions and the branch sites of arteries. Development of SFs is considered to be one of the morphological responses of ECs to mechanical stresses, and SFs appear to play an important role in maintaining cellular structural integrity by strengthening cell-to-matrix adhesion. We emphasize that these regional differences in endothelial HSP expression levels correlated with changes in SF arrangement. In ECs in the curved portions and the branch sites of rat arteries examined, high expression of HSPs was associated with significant changes in SF formation. Although the mechanisms by which HSPs modulate endothelial SFs are unknown, we have recently demonstrated that HSPs, especially HSP 70, probably regulate the induction of SF formation in ECs in some straight portions of rat arteries in response to mechanical stretch stresses[14]. Together,

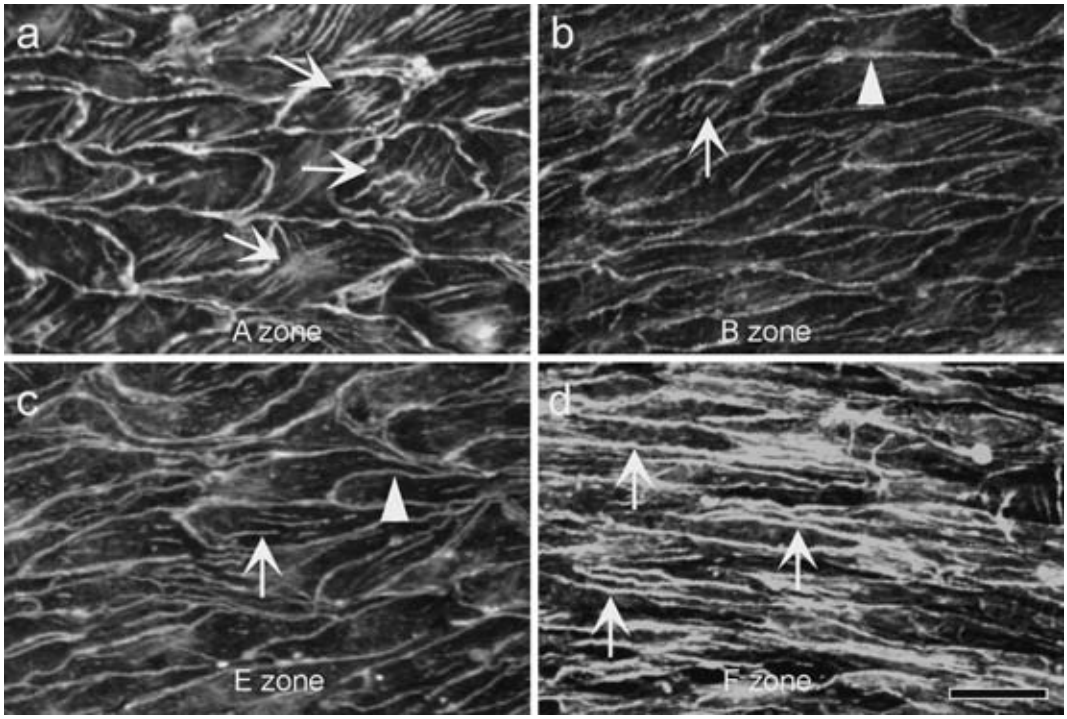

Fig. 4 Rhodamine-phallodin staining of ECs in zones A **a**, B **b**, E **c**, and F **d** shown in **Figure 1a–c**. In A zone **a**, the central SFs are randomly oriented **(arrows)**. In zones B **b** and E **c**, the ECs contain thin, short central SFs **(arrows)** and circumferential SFs **(arrow heads)**. In zone F **d**, the ECs have very long, thick central SFs **(arrows)** but lack circumferential SFs. The direction of blood flow is from left to right. Scale bar=20 μm.

these findings imply that vascular HSPs play an important role in the maintenance of vascular homeostasis via SF formation.

Several researchers have studied HSP expression in cultured cells *in vitro*[13,21–23]. However, few researchers have reported *in vivo* HSP expression data obtained using conventional histological sections that contain all three component layers of blood vessels (*i.e.*, tunica intima, media, and adventitia)[24,25]. Accurate evaluation of the *in vivo* expression and distribution of macromolecules in ECs may be more difficult because endothelial profiles in cross-sectioned vessels have extremely small volume. In the present study, we used EC sheets to perform histochemical analyses of HSP expression and SF formation in ECs under normal physiological conditions. Because the prepared samples were EC monolayers, IF microscopy with the EC sheet technique could clearly demonstrate the two-dimensional morphological and cytochemical dynamics of vascular endothelium *in vivo*.

Evidence for the involvement of HSPs and anti-HSP antibodies in the development of atherosclerotic vascular diseases is accumulating[26–29]. Antibodies against microbial HSPs produced in response to infection cross-react with HSPs in stressed ECs owing to the high degree of sequence homology that exists between human and microbial HSPs. This immunological cross-reaction causes endothelial damage and early atherosclerotic lesions. The arch, the orifices of the brachiocephalic trunk, the common carotid artery, the subclavia, the branch points of the mesenteric and renal arteries, and the iliac bifurcation are common early atherosclerotic lesions. In this study, we have shown that such atherosclerotic lesions already exhibit higher expression levels of HSP 60 and 70 under normal physiological conditions. Our findings indicate that these lesions can be readily affected by antibodies against microbial HSPs after disruption of vascular endothelial homeostasis because HSPs are enriched in ECs in these lesions. Many studies using experimental animal models indicate that HSP 60 and anti-HSP 60 may play important roles in the

initiation of atherogenesis. However, the roles of HSP 70 and anti-HSP 70 in cardiovascular diseases are still disputed. Chan et al.[30] have found a significant correlation between anti-HSP 70 antibody and different vascular diseases (i.e., lower limb claudicants, lower-limb critical ischaemia, and abdominal aortic aneurysms), suggesting that HSP 70 and anti-HSP 70 are involved in the development and progression of atherosclerosis. Although our study provides fundamental information for understanding HSP-cytoskeleton interactions in ECs, HSP expression and SF formation involved in stress responses under pathological (preferential atherogenic) conditions remain unclear. Recently, Foteinos et al.[31] have reported that in the presence of risk factors, e.g., hypercholesterolaemia, ECs overexpress HSP 60, and humoral and cellular immunity against HSP 60s damage ECs. To examine pathophysiological aspects of HSPs and SFs, we are performing further studies with the EC sheet technique.

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