HSP47 Expression by Smooth Muscle Cells Is Increased During Arterial Development and Lesion Formation and Is Inhibited by Fibrillar Collagen

Edward Rocnik, Laura Saward, J. Geoffrey Pickering

Abstract—HSP47 is a heat-shock protein that interacts with intracellular procollagen. It has been found in fibrous atherosclerotic plaque, but its involvement in acute vascular restructuring is unknown. We analyzed the expression of HSP47 and its regulation in the developing rat aorta and after balloon injury to the adult rat carotid artery. HSP47 was strongly expressed in each layer of the maturing fetal aorta (embryonic day 17 to birth). Expression declined during the first 4 postnatal days but persisted at low abundance into adulthood. HSP47 expression was substantially upregulated in the injured carotid artery, with intense immunostaining in neointimal smooth muscle cells (SMCs). HSP47 expression in SMCs was correlated with the emergence of a less mature phenotype and with expression of type I procollagen. Interestingly, a precipitous decline in HSP47 expression was evident during aortic development and after carotid artery injury, in association with the appearance of collagen fibrils in the local extracellular matrix. Furthermore, type I collagen fibrils, but not collagen monomers, inhibited expression of HSP47 by SMCs. These findings indicate that upregulation of HSP47 is a feature of vascular restructuring, including acute neointimal formation, and that the constituents of the extracellular matrix regulate the duration of expression. This feedback control may be important for self-termination of vascular development and lesion growth. (Arterioscler Thromb Vasc Biol. 2001;21:40-46.)

Key Words: smooth muscle cells • collagen • heat-shock protein • neointima • vascular development

The elaboration of collagen fibrils by vascular smooth muscle cells (SMCs) is vital to the structural and functional integrity of the artery wall. Collagen provides mechanical strength to the artery wall, sufficient to withstand the large hemodynamic loads imposed on it, and also serves as an important ligand that regulates SMC proliferation and migration.1–3

The major collagen species in both normal and diseased human arteries is type I collagen, a heterotrimeric, fibril-forming collagen that comprises 2 α1(I) collagen chains and 1 α2(I) collagen chain. The component α-chains are derived from precursors, namely proα1(I) collagen and proα2(I) collagen chains, that associate with each other in the lumen of the endoplasmic reticulum (ER). This assembly process requires precise sorting and folding of the procollagen chains within the ER and must occur before procollagen can be transported out of the cell. Quality control for procollagen assembly is dependent on a number of ER-resident enzymes and molecular chaperones. For example, prolyl 4-hydroxylase hydroxylates proline residues, the presence of which are necessary for winding of the long triple helical domain. The molecular chaperones, protein disulfide isomerase and immunoglobulin heavy-chain binding protein, transiently bind to a target region of the propeptide and facilitate either physiological folding or degradation of misfolded protein.4,5

HSP47 is a 47-kDa heat-shock-inducible glycoprotein that has also been found to associate with procollagen in the ER.6–7 Although its exact role in procollagen processing is unclear, data suggest that it acts as a collagen-specific chaperone. HSP47 has been localized exclusively to the ER of collagen-producing cells8 and has been found to associate with nascent type I procollagen chains as they translocate into the ER.6,9 It has also been shown to bind triple helical procollagen in the ER, where it may stabilize this conformation.10 Inhibition of HSP47 expression with antisense oligonucleotides has been associated with decreased expression of type I collagen in mouse fibroblasts9 and in a rat model of glomerulosclerosis.11

Recently, we determined that HSP47 was expressed by human arterial SMCs in culture, and we identified HSP47 in the fibrous cap of human atherosclerotic plaque.12 These findings highlight an involvement of this stress protein in vascular disease and, in particular, with fibrotic changes in the artery wall. The in vivo association between HSP47 expression and vascular fibrosis is consistent with other studies that have found abundant HSP47 in fibrotic organs but little, if any, in normal tissues.13–15 Thus, although the in vivo role for HSP47 is not well defined, the expression data to date suggest fibrotic repair as a dominant context. HSP47 has, however, also been detected during embryological development of the heart, lung, and kidneys.16–18

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developmental profile for arterial expression has not been defined; however, the data from other organs raise the possibility that HSP47 may have a broader role in arterial restructuring, beyond that of vascular scarring.

To investigate the possibility that HSP47 is involved in diverse forms of arterial remodeling, we analyzed HSP47 expression in the developing rat aorta and in the adult rat carotid artery during injury-induced neointima formation. Because SMC phenotype is a fundamental determinant of arterial restructuring, we also evaluated the relationship between expression of HSP47 and SMC differentiation status. Finally, we explored a mechanistic relationship between the termination of HSP47 expression by SMCs contributing to vascular restructuring and the appearance of nascent collagen fibrils in the extracellular space. The findings indicate that upregulation of HSP47 is a novel feature of acute vascular remodeling and that the duration of HSP47 expression under these circumstances is regulated by constituents of the extracellular matrix (ECM).

Methods

Antibodies and Reagents

Antibodies used included a mouse monoclonal antibody to rat HSP47 that we determined cross-reacts with porcine HSP47; a rabbit polyclonal antibody to the C-telopeptide region of the α(I) chain of human type I collagen, LF67 (gift of Dr L.W. Fisher, National Institute of Dental and Craniofacial Research, Bethesda, Md)20,21; and a mouse monoclonal antibody to human calponin (Sigma Chemical Co). Biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG was used as a secondary antibody for immunostaining. Vector SG peroxidase substrate was obtained from Vector Laboratories Inc, and 3,3′-diaminobenzidine (DAB) was obtained from Sigma. Human procollagen-I collagen cDNA was derived from H6777 (American Type Culture Collection, Manassas, Va) and cloned into pGEM3 (pSP3, gift from Dr C. Farrell, Amgen, Thousand Oaks, Calif). HSP47 mRNA expression was detected by using a plasmid containing a partial cDNA clone for rat HSP47 (pLP1).22 Rat tail collagen was isolated from tendons dissected from rat tails. Tendons were dissolved in 0.1 mol/L acetic acid at 4°C for 24 hours. Insoluble matter was removed, and NaCl was added to the solution to a final concentration of 1.7 mol/L to precipitate the collagen, which was retrieved by centrifugation at 3500g at 4°C for 20 minutes. The precipitate was resuspended in a minimal volume of 0.1 mol/L acetic acid and then reprecipitated with 1.7 mol/L NaCl. This precipitate was resuspended in 0.1 mol/L acetic acid and dialyzed against 17 μmol/L acetic acid for 3 to 5 days. The amount of collagen was quantified by Lowry assay (Bio-Rad) and the final concentration adjusted to 4 mg/mL with 17 μmol/L acetic acid.

Fetal, Neonatal, and Adult Animal Tissues

Timed-pregnant Sprague-Dawley rats (Charles River Canada, St. Constant, Quebec) were killed by anesthesia and subsequent asphyxiation with pentobarbital (40 mg/kg) and bupromorphine (0.05 mg/kg) subcutaneously and anesthetized with pentobarbital (40 mg/kg) intraperitoneally. The left carotid artery was injured using a 2F Fogarty catheter, as described.23 On designated days the animals were killed (pentobarbital, 110 mg/kg intraperitoneally), and the carotid arteries were perfused in situ with PBS and then perfusion-fixed with methanol– Carnoy’s fixative (methanol/chloroform/glacial acetic acid, 6:3:1 vol/vol/vol). The excised arteries were placed in the same fixative for an additional 3 hours and then embedded in paraffin.

Balloon Injury to the Rat Carotid Artery

Adult Sprague-Dawley rats were pretreated with atropine (0.04 mg/kg) and buprenorphine (0.05 mg/kg) subcutaneously and anesthetized with pentobarbital (40 mg/kg) intraperitoneally. The left carotid artery was injured using a 2F Fogarty catheter, as described.23 On designated days the animals were killed (pentobarbital, 110 mg/kg intraperitoneally), and the carotid arteries were perfused in situ with PBS and then perfusion-fixed with methanol– Carnoy’s fixative (methanol/chloroform/glacial acetic acid, 6:3:1 vol/vol/vol). The excised arteries were placed in the same fixative for an additional 3 hours and then embedded in paraffin.

Immunohistochemistry

Deparaffinized tissue sections (4 μm) were subjected to antigen retrieval by immersion in 10 mol/L sodium citrate buffer, pH 6.0; microwaving on full power for 2.5 minutes and then on low power for 7 minutes; and maintained afterward in hot buffer for 20 minutes. Nonspecific binding of primary antibodies was blocked with 10% horse serum or 10% goat serum in PBS. Primary antibodies in blocking solution were incubated with sections overnight at 4°C. Endogenous peroxidase activity was inhibited with 3% H2O2 in methanol. Sections were incubated with biotinylated secondary antibody for 1 hour at room temperature. Bound antibody was detected with the ABC Elite kit from Vector Laboratories and visualized with DAB. The sections were counterstained with Harris’ hematoxylin. Formalin-fixed sections of human dermal granulation tissue served as a positive control for HSP47.

Collagen Fibril Assessment by Circular Polarization Microscopy

To visualize polymerized collagen fibrils in the extracellular space, a polarized light microscopy approach was taken. Deparaffinized 4-μm-thick sections were immersed in picrosirus red solution (1% aqueous F3BA Sirius red and water-saturated picric acid, 1.9 vol/vol) for 60 minutes. The sections were then dehydrated and coveredipped. The sections were viewed under circularly polarized light by using a Nikon Optiphot microscope, customized for circular polarization optics with quarter-wave plates inserted in the light path both below the polarizer and above the analyzer.

Western Blot Analysis

Cultured SMCs were harvested in lysis solution (0.1 mmol/L EDTA, 1% wt/vol SDS, 1% wt/vol sodium deoxycholate, and 0.1% Triton X-100 in PBS, pH 7.4) with PMSF (0.1 mmol/L) and leupeptin (10 μg/mL). Carotid arteries were homogenized in the same solution after the adventitia had been stripped away under microscopic guidance. Equal amounts of protein were resolved on 6% (for collagen) or 12% (for HSP47 and calponin) polyacrylamide gels and evaluated by Western blot analysis, as described previously.23

Northern Blot Analysis

Porcine SMCs in the third through fifth subcultures were plated onto culture dishes precoated with monomeric collagen or in 3-dimensional collagen fibril lattices. Monomeric collagen–coated dishes were prepared by washing dishes with 0.1% acetic acid for 1 hour at room temperature and then coating them for 3 hours with rat tail collagen diluted in 0.1% acetic acid to 100 μg/mL. For the collagen lattice, SMCs were suspended at 2.5 × 104 cells/mL in medium 199 supplemented with 4% fetal bovine serum. The collagen lattice was made by mixing 4 mL of the cell suspension with 80 μL of 1N NaOH, 0.4 mL of 10× minimum essential medium (Gibco), 2 mL of rat tail collagen (4 mg/mL), and 1.52 mL of 0.1% acetic acid. This mixture was plated in a 100-mm tissue-culture dish. Total RNA was isolated (TRIzol reagent, Gibco) and Northern blots were prepared as previously described.23 Membranes were exposed to a phosphor imager screen (Molecular Dynamics), and band density was quantified and expressed relative to that of 18S rRNA.

Results

HSP47 Is Abundantly Expressed in the Developing Rat Aorta and Weakly Expressed in the Adult Rat Aorta

Aortas from fetal, newborn, and adult rats were studied for HSP47 expression by immunohistochemistry. We chose embryonic day 17 as the first time point for study because a multilayered aortic wall has formed by this time but the ECM remains relatively undeveloped, as evidenced by the lack of discrete elastic lamellae. Furthermore, this time point precedes the well-documented perinatal surge in procollagen expression in cardiovascular tissue.26 As shown in Figure 1A, there was abundant and diffuse HSP47 immunoreactivity...
throughout the aortic wall at embryonic day 17. By day 19 of gestation, the aorta was larger, with better delineation of the 3 aortic layers such that HSP47 expression could be localized to endothelial cells, medial SMCs, and adventitial cells (Figure 1B). Expression in all 3 aortic layers was also evident on the first neonatal day; however, the level had declined in the innermost layers of the media (Figure 1C). This pattern was also observed in 4-day-old aortas (Figure 1D). Five months after birth, expression of HSP47 was substantially reduced though still detectable in the endothelium, medial SMCs, and adventitial cells (Figure 1E).

To relate the expression profile of HSP47 to the accumulation and organization of collagen fibers, we exploited the fact that polymerized, fibrillar collagen is birefringent and thus visible by polarized light microscopy. When stained with picrosirius red and illuminated by circular polarization optics, nascent collagen fibrils/fibers can be identified with high sensitivity and specificity.27,28 As shown in Figures 1F and 1G, by embryonic days 17 and 19, collagen fibers were evident in the adventitial layer only, despite the abundance of HSP47 throughout the artery wall at these times. Collagen fiber formation in the adventitia notably progressed by 1 and 4 days after birth, with a well-developed fibrillar network. At these neonatal times, short collagen fibrils were evident in the media; however, these were scant and weakly birefringent. By 5 months, however, collagen fibrils were well developed in the media. A band of birefringent collagen bundles was evident subjacent to the internal elastic lamina, and a pair of collagen fiber bands straddled each medial elastic lamella.

**HSP47 Expression Is Rapidly Increased During Injury-Induced Neointimal Formation**

To determine whether HSP47 expression was also upregulated during acute arterial remodeling/repair of the adult artery, the left carotid artery of rats was subjected to balloon injury. As illustrated in Figure 2A, in uninjured carotid arteries fixed with methanol–Carnoy’s reagent, there was a weak signal for HSP47 in SMCs, consistent with the pattern observed in the aorta of adult rats. HSP47 was also detected in adventitial cells. HSP47 expression was less obvious in the endothelial cells, although this was fixation dependent because endothelial cell staining was evident in carotid arteries fixed with 4% paraformaldehyde (data not shown). Four days after injury, HSP47 was strongly expressed in SMCs of the primordial neointima, as well as in SMCs within the inner layers of the media (Figure 2B). At 14 days, there was intense cellular staining throughout the expanded neointima, as well as in cells of the inner media and in adventitial cells (Figure 2C). By 28 days, HSP47 expression levels had declined notably, with weaker expression in scattered intimal cells (Figure 2D).

Collagen fibril formation was assessed in adjacent sections by using polarization microscopy. As shown in Figures 2E through 2G, birefringent fibers were detectable in the neointima by day 14, although they were thin, loosely organized, and evident only in the deeper regions. By day 28 (Figure 2H), at which time HSP47 expression was relatively low, there was a relative abundance of thicker and more densely packed fibrils throughout the neointima (Figure 2H).

**HSP47 Marks Less-Differentiated SMCs**

To assess the relationship between HSP47 and calponin expression in vivo, control and balloon-injured rat carotid arteries were assessed by Western blot analysis after the adventitia had been removed. As shown in Figure 3, calponin expression underwent a striking decline 6 days after injury, consistent with the emergence of a less-differentiated SMC phenotype. This was followed by a gradual increase in calponin abundance at 14 and 28 days. In contrast, HSP47 expression, which was detectable at low levels in the uninjured artery, increased substantially 6 days after injury and declined thereafter. Type I procollagen expression was not detectable in the uninjured artery but displayed a transient rise after injury, similar to HSP47. A similar relationship between HSP47 expression and SMC differentiation status was established by tracking SMCs in vitro after their acute dispersion from the porcine carotid artery. An 8-fold increase in HSP47 coincided with the initiation of DNA synthesis and decline in calponin expression (Figure 1; published online at http://atvb.ahajournals.org).

**HSP47 Expression by SMCs Is Inhibited by a Polymerized, 3-Dimensional Collagen Lattice**

The in vivo expression profile of HSP47, during both vascular development and vascular repair, was inversely correlated with the emergence of collagen fibrils in the ECM. To determine whether collagen in either its monomeric or fibrillar form could in fact regulate HSP47 expression by SMCs, they were cultured either on monomeric type I collagen or in a 3-dimensional collagen fibril lattice. As shown in Figure 4, expression of HSP47 mRNA by SMCs within a fibrillar collagen lattice fell to 0.4 of basal levels within 24 hours. In contrast, expression of HSP47 in SMCs cultured on monomeric collagen declined minimally. Consistent with previous reports, polymerized collagen also inhibited expression of proα(I) collagen mRNA levels in SMCs. After 24 hours of stimulation with fibrillar collagen, proα(I) collagen mRNA levels fell to 0.2 of basal levels. A more modest decline was observed for SMCs on monomeric collagen (0.5 of basal level).

**Discussion**

HSP47 is a stress protein that appears to act as a molecular chaperone during intracellular processing of procollagen.7 Insight into the in vivo relevance of HSP47 has largely come from its identification in organs undergoing fibrosis.13–15 A relationship between HSP47 expression and fibrosis has been identified in human coronary arteries, where HSP47 was localized to the fibrous cap of atherosclerotic plaque.12 The current studies indicate, however, that HSP47 expression in the artery wall is not confined to fibrotic conditions. Rather, HSP47 was detected in abundance during aortic development and during the early stages of neointima formation after vascular injury. In addition, HSP47 was expressed constitutively, at a low level, in the healthy adult artery wall. These findings suggest a broad involvement of HSP47 in the artery wall both physiologically and pathologically.

The late fetal and early postpartum period is characterized by a surge in ECM deposition in the vasculature. Collagen content has been recognized to rapidly increase after birth in the rat and sheep aorta.29,30 At the gene expression level, proα1(I) collagen
Figure 1. Photomicrographs of the embryonic and adult rat thoracic aorta showing expression of HSP47 (A–E) and the associated presence of birefringent collagen fibrils (F–J). HSP47 expression was assessed by immunostaining with a monoclonal antibody to HSP47 and visualized by using DAB substrate (brown color). Collagen fibrils were imaged in nearby sections by circular polarization microscopy of tissue sections stained with picrosirius red. Tissues were harvested on day 17 of gestation (A, F), day 19 of gestation (B, G), day 1 after birth (C, H), day 4 after birth (D, I), and at 5 months of age (E, J).
Figure 2. Photomicrographs of normal and balloon-injured left carotid artery of the rat showing expression of HSP47 (A–D) and the associated presence of birefringent collagen fibrils (E–H). HSP47 expression was assessed by immunostaining, and collagen fibrils were imaged in adjacent sections by circular polarization microscopy, as described. Arteries were harvested uninjured (A, E) as well as 4 days after injury (B, F), 14 days after injury (C, G), and 28 days after injury (D, H). HSP47 expression is weakly evident in the normal artery wall, strongly evident in the neointima at 4 and 14 days, and weakly evident by 28 days. Thin collagen fibrils become apparent in the neointima by 14 days and abundant at 28 days. Arrows identify the intima-media junction.
HSP47 expression was found to substantially increase in the rat coronary artery immediately after birth.49 Our finding of HSP47 expression in the late stages of embryonic development and in the first few neonatal days is thus consistent with the concept that HSP47 is a participant in procollagen processing in vivo. Furthermore, it suggests that the intracellular machinery for optimizing procollagen production is in place at the time of the perinatal surge in collagen production.

HSP47 expression also increased shortly after vascular injury. As with aortic development, the early stages of artery repair and neointimal formation after balloon injury have been associated with collagen production. Abundance of proα2(I) collagen mRNA was found to be markedly increased 7 days after balloon injury to the rat carotid artery,10 and proα1(I) collagen mRNA and total collagen synthesis rates were found to increase 1 week after balloon injury to rabbit iliac arteries.32 Our observation of type I procollagen peptides are detectable in the vessel wall 6 days after balloon injury further indicates that the early stages of neointima formation entail increased fibrillar collagen synthesis. This in vivo relationship between HSP47 expression and procollagen production in diverse contexts provides strong support to the hypothesis that HSP47 plays an important role in collagen metabolism by SMCs.

The pronounced increase in HSP47 in the primordial neointima and inner medial SMCs early (4 days) after balloon injury suggests that HSP47 expression is correlated with the presence of less-differentiated SMCs. This was verified by documenting a reciprocating relationship between HSP47 expression and that of calponin, a marker of relatively mature SMCs, after balloon injury. HSP47 expression also increased in newly cultured SMCs acutely dispersed from the porcine carotid artery, consistent with the initiation of SMC DNA synthesis. Thus, regardless of its role in procollagen processing, HSP47 expression appears to be a marker of less-differentiated SMCs, including those SMCs that manifest increased ECM synthesis.

The finding that HSP47 was constitutively expressed at a low level in the adult rat aorta and carotid artery was not predicted. Prior studies of HSP47 expression in rats 69 days after birth found little, if any, HSP47 mRNA or protein in homogenates of rat heart, kidney, or lung.16,17 Similarly, in an assessment of human coronary arteries, we did not detect HSP47 expression by immunohistochemistry in normal arteries.12 Because the only available antibody to HSP47 is raised against rat HSP47, low-level expression in the human artery wall may not be optimally immunodetectable. The basal expression of HSP47 observed in the current study of rat arteries was further noteworthy because it was even evident in endothelial cells, which are generally considered to express little, if any, collagen under normal conditions. The presence of constitutively expressed HSP47 could suggest that there is in fact a very low level of collagen turnover in the normal aorta and carotid artery. Alternatively, it may suggest that HSP47 and collagen production are not inextricably linked. In this regard, it is noteworthy that HSP47 expression has been found to be increased by certain stresses, including heat and oxidized LDL, without a concomitant increase in type I collagen expression.6,12

The increases in HSP47 expression observed during vascular development and repair were transient. During aortic development, HSP47 expression fell during the first few neonatal days. After vascular injury it declined between 14 and 28 days. In both cases, the relatively precipitous decline in HSP47 expression was correlated with the early accumulation of polymerized collagen fibrils in the artery wall. Although traditionally difficult to visualize, these early collagen fibrils were identified on the basis of their acquisition of a birefringent structure. The findings thus established that HSP47 expression is inhibited as soon as or shortly after de novo collagen fibrils are formed in the local ECM. Furthermore, the in vitro studies suggested that these newly polymerized fibrils were in fact responsible for the downregulation of HSP47 expression.

Polymerized collagen fibrils have previously been shown to regulate gene expression in several cell types.2,34 Interestingly, many of the genes regulated by fibrillar collagen encode proteins that are intimately involved in collagen fibril turnover. In fibroblasts, fibrillar collagen has been shown to induce the expression of collagenase-135 and collagenase-3,36 increase expression of the collagen-binding α2β1 integrin,37 and inhibit the expression of type I collagen.38 The current finding that fibrillar, but not monomeric, collagen inhibits expression of HSP47 by SMCs thus not only identifies a new gene target for fibrillar collagen but also strengthens the paradigm that the abundance and architecture of collagen fibrils provide feedback to the SMCs responsible for remodeling the collagen matrix. Because abundant collagen deposition is typically a late stage of tissue remodeling, the downregulation of HSP47 expression by colla-
gen fibrils may be integral to a mechanism that effectively terminates the remodeling processes itself.

In summary, we have determined that the stress protein HSP47 is abundantly expressed in the artery wall under diverse circumstances of restructuring, including acute neointimal formation. In these contexts, HSP47 expression was closely linked to the emergence of less-mature SMCs. Furthermore, the findings indicate that HSP47 expression by SMCs can be inhibited by newly polymerized collagen fibrils in the extracellular space, implicating a novel feedback mechanism that may be critical to self-termination of vascular development and lesion growth.

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Figure I legend:

Western blots showing expression of calponin, Hsp47, and type I procollagen following balloon-injury to the rat carotid artery. The left carotid artery was harvested before and at designated times following balloon injury and analyzed as described in Methods. The bands detected by the anti-type I collagen antibody LF67 (bottom blot) include procα1(I) collagen chain, partially processed procα1(I) collagen chain lacking either the NH₂ or COOH-terminal propeptides (procα1(I) collagen N-/C-) and processed α1(I) collagen that either preexisted in the artery wall or was newly deposited.