Biological Significance of Decreased HSP27 in Human Atherosclerosis

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Objective—Because culprit atherosclerotic plaques contain proteases, we hypothesized that the diminished heat shock protein 27 (HSP27) released by atherosclerotic plaques could be due to proteolysis. We assessed the role of HSP27 in human vascular smooth muscle cells (VSMCs) under proteolytic injury.

Methods and Results—Active plasmin is present in culprit atherosclerotic plaques. Recombinant HSP27 was cleaved by plasmin and this effect was prevented by different inhibitors. Fragments and aggregated forms of HSP27 appeared after incubation of mammary control endarteries with plasmin. Coincubation of atherosclerotic plaques with recombinant HSP27 or mammary endarteries led to HSP27 proteolysis. After incubation of VSMCs with plasmin, HSP27 was overexpressed, phosphorylated, aggregated, and redistributed from the cytoskeleton to the cytosol, nucleus, and cell membrane. Plasmin-induced VSMC apoptosis was significantly higher in VSMCs treated by HSP27 siRNA. Immunohistochemical analysis of atherosclerotic plaques showed that plasmin(ogen) and apoptotic cells are localized in the core/shoulder whereas HSP27 and VSMCs are mainly expressed in the cap/media.

Conclusions—Extracellular HSP27 can be degraded by enzymes released from atherosclerotic plaques and may reflect a proteolytic imbalance. Intracellular HSP27 downregulation decreases VSMCs resistance to proteolytically-induced apoptosis. HSP27 might play a pivotal role in the prevention of plaque instability and rupture.

Key Words: anoikis ■ atherosclerosis ■ HSP27 ■ plasmin ■ proteases
of HSP27 in the extracellular and intracellular compartments under proteolytic injury is addressed.

Methods

Reactives

Low-endotoxin human HSP27 recombinant protein was purchased from Stressgen (ESP-175). Human plasmin was from American Diagnostica, aprotinin from Sigma, and VFK from Calbiochem.

Tissue Culture

Human carotid samples were obtained from patients undergoing carotid endarterectomy and nonatherosclerotic endarteries (mammary) were obtained from patients undergoing cardiac surgery in the Centre Cardiologique du Nord (Saint-Denis, France). These tissues are considered as surgical waste in accordance with French ethical laws (L.1211-3 to L.1211-o 9) and the INSERM Ethics Committee. Samples were washed to remove residual blood. After macroscopic evaluation by a trained vascular surgeon (J.B.M.), carotid endarterectomy samples were dissected as previously described,1,2 separating the stenosing culprit plaque (CP) with intraplaque hemorrhage from the noncomplicated fibrous adjacent area (NCP), mainly composed of VSMCs.

Seventeen CPs and NCPs tissue samples (~15 mg) were incubated in Tris/HCl, pH 7.4, 100 mmol/L NaCl, 0.01% Tween 20 with the plasmin substrate Tosyl-Gly-Pro Arg-Mca (5 μmol/L; Sigma).20 Substrate hydrolysis was monitored for 2 hours by spectrophotometry (Hitachi F-2000; Exc. 347 nm Em.440 nm).

When indicated, CP was divided into similar pieces (~15 mg) and incubated with 25 μL of RPMI containing 1 μg of recombinant HSP27 in the presence or absence of aprotinin overnight at 37°C. Equal segments of mammary endarteries were incubated with or without plasmin in the presence or absence of aprotinin for 24 hours in serum-free RPMI medium at 37°C. Conditioned media were collected and separated by a 12.5% SDS-PAGE as described below. In some cases, after incubation of mammary endarteries with plasmin, the tissue was fixed in paraformaldehyde and embedded in paraffin for detection of apoptosis as described.

Enzyme-Linked Immunosorbent Assay

Soluble HSP27 levels were measured in the conditioned media of human VSMCs after different experimental conditions with a commercially available kit (Oncogene) following the manufacturer’s procedure as described.2

Western Blot

Equal amounts of proteins were separated by a 12.5% SDS-PAGE. The gel was transblotted onto a nitrocellulose membrane, blocked with 10% nonfat milk in TBS-T (TRIS-buffered saline pH 7.4 to 0.1% Tween 20) for 1 hour, and then incubated with mouse anti-HSP27 antibody (1:5000 dilution, Calbiochem) or rabbit monoclonal phospho-HSP27Ser28 (1:5000, Epitomics). Membranes were then washed with TBS-T and incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (1:10 000 dilution) for 1 hour. After 5 washes, the signal was detected using the chemiluminescence kit (Amersham).

Compartmentalization

For isolation of cytoplasmic, nuclear, membrane and cytoskeletal proteins in the different experimental conditions, the Compartmental Protein Extraction Kit (Chemicon) was used following the manufacturer’s instructions.

Transfection of Small Interfering RNA

Human VSMCs were grown to 50% confluence in 12-well plates and transfected with a mixture composed of 2.5 mol/L CaCl2, 25 nmol small interfering RNA (siRNA) (human HSP27 siRNA sc-29350; Santa Cruz Biotechnology) and calcium phosphate buffer (280 mmol NaCl, 1.5 mmol Na2HPO4, 12 mmol/L glucose, 10 mmol/L KCl, 50 mmol/L Hepes, pH7) in 10% fetal calf serum complete medium. After 18 hours, cells were washed and cultured for 72 hours in complete medium, and serum-depleted for 24 hours before addition of plasmin.

Cell Detachment Assay

Human VSMCs were grown to confluence in 12-well plates and serum-deprived for 24 hours before stimulation. At the end of the experiment, the cells were washed with phosphate-buffered saline. Remaining viable adherent cells were assessed using the MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide).16

Measurement of DNA Fragmentation In Vitro

Histone-associated DNA fragments were quantified by using a photometric enzyme immunoassay (Cell death detection ELISAPLUS; Roche) following the manufacturer’s instructions.17

Immunocytofluorescence

Human VSMCs were grown on glass slides (LABTEK). Cells were washed and fixed in cold methanol and paraformaldehyde for 10 minutes each and then incubated with 6% goat serum in phosphate-buffered saline for 1 hour at room temperature. Mouse anti-human HSP27 (1/500 dilution, Calbiochem) was used as primary antibody and an Alexa 488-labeled goat anti-mouse immunoglobulin G was used as secondary antibody (1/5000 dilution; Molecular Probes). Controls were incubated with corresponding naive immunoglobulins. After extensive washing, cells were mounted in 70% glycerol in phosphate-buffered saline.

Immunohistochemistry

Specimens were fixed with paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5-μm-thick sections using an anti-active caspase-3 antibody (G7481, Promega) and a monoclonal antibody to single stranded DNA (Apostain; Alexis) as markers of apoptosis in situ, an anti-plasmin(ogen) antibody (which recognizes both plasmin and plasminogen; Technoclone), a goat anti-human HSP27 antibody (SC-1049; Santa Cruz Biotechnology), and an anti-smooth muscle alpha actin antibody (Dako) as described.21,22

Quantification

Preparations were digitalized at 400× magnification, and the entire carotid section was reconstituted with Cartograph and then analyzed with Histolab software (Microvision Instruments, France). Computer-assisted morphometric analysis was performed by circling the different atherosclerotic regions: cap, core, media, and shoulder. Results were first collected as means of gray values per mm² for each region and then expressed, for each antibody, as percentage of total staining per carotid (100% = cap + core + media + shoulder).

Statistical Analysis

Statistical analysis was performed with GraphPAD InStat (GraphPAD Software). All experiments were performed at least 3 times. Results are expressed as mean±SD and were analyzed by ANOVA (differences were considered significant when p<0.05). Differences between noncomplicated and culprit plaques were analyzed by the Wilcoxon paired test.

Results

Active Plasmin Is Present in Culprit Atherosclerotic Plaques

We recently showed that HSP27 levels are decreased in conditioned media of culprit atherosclerotic plaques (CP) in comparison to those of control arteries.2 Because CP contain proteases,18,19 we hypothesized that the decreased HSP27 released by atherosclerotic plaques could be caused by proteolysis of the protein. Moreover, CP contained active
plasmin in higher amounts than their corresponding NCP (P<0.001).

HSP27 Is Proteolyzed by Plasmin

Recombinant HSP27 (0.04 μg) was incubated with plasmin (31.25 to 125 nmol) for 15 to 60 minutes at 37°C. Plasmin dose-dependently cleaved HSP27 (not shown). In addition, when plasmin was incubated with recombinant HSP27 for 15 minutes in the presence of different inhibitors (VFK and aprotinin), this effect was prevented (Figure 1a).

To assess whether HSP27 proteolysis shown in vitro could also occur in vivo, mammary arteries (used as a source of HSP27) were incubated overnight in the presence of 125 nmol plasmin. HSP27 released by mammary arteries was proteolyzed, obtaining a similar band to that observed after incubation of plasmin and recombinant HSP27 in vitro (Figure 1b). Moreover, a higher molecular weight band was detected corresponding to an aggregated form of the protein. This effect was partially prevented by aprotinin (Figure 1b).

HSP27 Levels in VSMCs After Incubation With Plasmin

In a next step, we sought to explore the potential biological significance of HSP27 degradation by proteolytic injury. First, we analyzed the effect of plasmin on HSP27 levels in human VSMCs in vitro. We were able to show by enzyme-linked immunosorbent assay that basal human VSMCs release HSP27 into the medium. It is noteworthy that levels of soluble HSP27 in VSMC supernatants are 10-times lower than those previously found in mammary conditioned medium determined by the same technique.2 Addition of plasmin for 60 minutes drastically decreased soluble HSP27 (6.5±3.5 ng/mL on basal cells versus 0.8±1.1 ng/mL on cells incubated 60 minutes with plasmin, P<0.05). Interestingly, HSP27 reappeared in the VSMC conditioned medium after 18 hour of incubation with plasmin (5.2±1.1 ng/mL). Therefore, we tested the possibility that VSMCs could synthesize and release HSP27 after plasmin stimulation as a defense
Because it has been previously shown that HSP27 is upregulated and phosphorylated in response to stress to ensure the maintenance of cell homeostasis and survival. We show here that plasmin (62.5 nM) increased HSP27 expression and phosphorylation (Figure 2a). It has been proposed that HSP27 phosphorylation lead to redistribution of HSP27 and the formation of dimers. After incubation with plasmin, we observed an upper band which is not present either in basal conditions or when cells lysates were subjected to SDS-PAGE reducing conditions, which could represent an aggregated form of the protein (dimers). However, no fragments of HSP27 could be detected suggesting that plasmin cannot directly cleave intracellular HSP27.

It is known that HSPs are redistributed within the cell after stress, and this translocation seems to be crucial for these proteins to play their protective role. We have analyzed the localization of HSP27 after stimulation by plasmin, we observed an upper band which is not present either in basal conditions or when cells lysates were subjected to SDS-PAGE reducing conditions, which could represent an aggregated form of the protein (dimers). However, no fragments of HSP27 could be detected suggesting that plasmin cannot directly cleave intracellular HSP27.

**Effect of HSP27 on Vascular Apoptosis**

HSPs provide a defense mechanism against external stressors, such as heat shock, oxidative stress, or mechanical stress. We thus investigated the potential role of HSP27 in plasmin-induced anoikis of human VSMCs by diminishing its expression using specific siRNA or adding exogenous HSP27 before incubation with plasmin.

Cells were transfected with calcium phosphate-containing or not-containing siRNA directed against HSP27 mRNA. After 72 hours, HSP27 protein levels are reduced by HSP27 siRNA treatment relative to control cells transfected with medium alone. HSP70 levels assessed by Western blot were equivalent in all conditions showing the specificity of the HSP27 siRNA used (Figure 3a). These results were confirmed by HSP27 immunofluorescence (Figure 3b). VSMCs were then incubated with 62.5 nmol plasmin for an additional 18 hours. Plasmin induced cell detachment (Figure 3c) and this effect was reversed by aprotinin (data not shown). In these conditions, VSMC viability was significantly reduced in siRNA-HSP27–treated cells versus control cells incubated with plasmin for 18 hour in nontransfected cells (upper panel) or in cells transfected with 25 nmol HSP27-siRNA (lower panel).

Because intracellular HSP27 is known to be anti-apoptotic, we checked whether the decreased VSMC viability after incubation with plasmin in siRNA-HSP27–treated cells was caused by an increase in VSMC apoptosis. Plasmin-induced VSMC apoptosis was increased 2-fold in HSP27 siRNA-treated cells (15.2±0.8 versus 32.5±1.3 A₄₀₅*10⁻³ min⁻¹,
P/H110210.05; Figure 4b). These data support an antiapoptotic role of intracellular HSP27 in VSMCs under protease-stress conditions. Moreover, ex vivo incubation of mammary endarteries with plasmin led to VSMC apoptosis, detectable within the tissue (Figure 4c).

In a second step, we analyzed the levels and localization of a marker of apoptosis in vivo such as active caspase-3 and that of HSP27, VSMCs, and plasmin(ogen) within atherosclerotic plaques. We show that HSP27 was mainly present in the cap and media, colocalizing with α-actin–positive VSMCs (Figure 5a, 5b), whereas active caspase 3 staining was localized in the core/shoulder regions of the plaques (Figure 5c). Interestingly, plasmin(ogen) is mainly stored within the core (Figure 5d), where most of apoptotic cells and lowest levels of HSP27 were found (Figure 5e). Taken together, these results suggest that HSP27 could be involved in the regulation of apoptosis in the atherosclerotic process.

**Discussion**

In a previous study, we validated an original approach analyzing the secreted proteins from atherosclerotic plaques and nonpathological arterial wall. More recently, using a similar procedure, we have identified HSP27, for which production by the arterial wall correlates negatively with atherosclerotic plaque complexity. Conversely, we show herein that culprit atherosclerotic plaques contain active plasmin in greater amounts than control samples. In the light of these results, we hypothesized that the diminished levels of HSP27 observed in atherosclerotic plaque supernatants could be due to its proteolysis. Indeed, we have shown in vitro that plasmin is able to degrade HSP27, either recombinant or present in conditioned medium of normal arteries or VSMCs. Furthermore, when recombinant or HSP27-rich conditioned medium were incubated with culprit atherosclerotic plaques in culture (as a source of proteases), similar bands of degradation were observed, supporting the fact that proteases secreted by atherosclerotic plaques could be responsible for the degradation of soluble HSP27 in vivo. Taken together, these data suggest that reduced HSP27 levels could reflect proteolytic imbalance occurring during pathological vascular remodeling process and may thus provide an index of plaque instability and rupture.

Intracellular HSPs provide the cells with a mechanism of defense against external stressors, such as heat shock, oxidative, or mechanical stress. Upregulation of the synthesis of some of these proteins ensures the maintenance of cell...
homeostasis and survival. We show that after incubation of VSMCs with plasmin, HSP27 is overexpressed and phosphorylated. It has been previously demonstrated that in human monocytes, plasmin can stimulate p38 mitogen-activated protein kinase (MAPK) signaling. Moreover, it has been shown that when this kinase cascade is activated, HSP27 is phosphorylated in response to stress. HSP27 phosphorylation leads to the formation of dimers. In agreement, we observed a ~54-kDa band of phosphorylated HSP27 after incubation of VSMC with plasmin, not present in the basal conditions and disappearing under reducing conditions, which could represent an aggregated form of the protein (dimers). It has been proposed that phosphorylation-induced changes in the structure of HSP27 molecules could regulate its biochemical activities, among them the stabilization of actin filaments. For example, HSP27 phosphorylation is involved in the protection of renal epithelial cells against cell detachment and apoptosis caused by nephrotoxic cellular stress. We previously reported that phosphorylated HSP27 (ser 82) is present in large amounts in the conditioned medium of undiseased endarteries and almost absent in atherosclerotic conditioned media.

In addition, it is known that HSP redistribution within the cell after stress is pivotal to the protective properties of these proteins. Small HSPs, such as HSP27, are translocated to the nucleus after stress. In agreement, we have shown that HSP27 translocates to the nucleus, but also to the cytosol and cell membrane after plasmin stimulation. Membrane-associated HSP27 may protect against the stress-induced damage of the cell membrane, as previously shown for other small HSPs. However, it has been suggested that this protective function of HSPs may be overridden in cases of massive stress or of chronic disease, such as atherosclerosis. HSP27 could contribute to actin filament stabilization when organized in small phosphorylated oligomers. In basal conditions, we have observed that HSP27 is localized in the cytoskeleton of VSMCs. After addition of plasmin, HSP27 is decreased in this fraction, which could reflect actin network disorganization associated with loss of cell anchorage and tensility.

Anoikis is defined as programmed cell death induced by the loss of cell/matrix interactions and is probably involved in pathological remodeling of vascular tissues, including de-endothelialization and plaque rupture in atherosclerosis, and VSMC disappearance in aneurysms. We have shown previously that plasmin can be generated in situ at the surface of the cells and be responsible for their anoikis by degrading pericellular adhesive glycoproteins. We showed that plasmin was able to induce VSMC detachment leading to the morphological and biochemical changes characteristic of anoikis. Little is known about the potential role of HSPs in anoikis. Here we have shown that downregulation (siRNA) of HSP27 enhanced the deleterious effect of plasmin on cell viability due to an augmented VSMC apoptosis.

Apoptosis of VSMCs in vivo is involved in the development of atherothrombosis and plays a major role in weakening the fibrous cap of the atheroma. VSMCs are dependent on survival signals coming from the extracellular matrix generating tensility within the cell. By degrading extracellular matrix components, proteases could lead to the disruption of these survival signals and eventually trigger programmed cell death. In this respect, several studies suggest the involvement of the plasminogen activation system in atherosclerosis. Our in vitro data suggest that intracellular HSP27, by modulating the effect of plasmin and other extracellular potential mediators of VSMC apoptosis, could favor the stability of atherosclerotic plaques in vivo. We show that apoptotic cells are localized in the shoulder and core of the plaque, where most of the plasminogen is stored. The plasmin formed at the contact of cells expressing plasminogen activators could be responsible for HSP27 degradation, pericellular proteolysis, and cell death participating in the weakening of the plaque.

In conclusion, extracellular degradation of HSP27 in atherosclerotic plaques could reflect a pathological vascular remodeling process, in which the final balance between proteases and antiproteases favors the degradative process of the extracellular matrix. In addition, our in vitro results suggest that intracellular HSP27 could have an atheroprotective role in the vascular wall by diminishing VSMC apoptosis, a determinant of plaque instability.

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