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The regulatory mechanism of Hsp90α secretion from endothelial cells and its role in angiogenesis during wound healing

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ABSTRACT

Heat shock protein 90α (Hsp90α) is a ubiquitously expressed molecular chaperone, which is essential for the maintenance of eukaryote homeostasis. Hsp90α can also be secreted extracellularly and is associated with several physiological and pathological processes including wound healing, cancer, infectious diseases and diabetes. Angiogenesis, defined as the sprouting of new blood vessels from pre-existing capillaries *via* endothelial cell proliferation and migration, commonly occurs in and contributes to the above mentioned processes. However, the secretion of Hsp90α from endothelial cells and also its function in angiogenesis are still unclear. Here we investigated the role of extracellular Hsp90α in angiogenesis using dermal endothelial cells in vitro and a wound healing model in vivo. We find that the secretion of Hsp90α but not Hsp90β is increased in activated endothelial cells with the induction of angiogenic factors and matrix proteins. Secreted Hsp90α localizes on the leading edge of endothelial cells and promotes their angiogenic activities, whereas Hsp90α neutralizing antibodies reverse the effect. Furthermore, using a mouse skin wound healing model in vivo, we demonstrate that extracellular Hsp90α localizes on blood vessels in granulation tissues of wounded skin and promotes angiogenesis during wound healing. Taken together, our study reveals that Hsp90α can be secreted by activated endothelial cells and is a positive regulator of angiogenesis, suggesting the potential application of Hsp90α as a stimulator for wound repair.

1. Introduction

The 90-kDa heat shock protein (Hsp90) is a universally expressed and abundant molecular chaperone. It is essential for the activation and stability of a selected group of cellular proteins [1]. A remarkable proportion of Hsp90 substrates, or client proteins, are kinases and transcription factors involved in signal transduction [2]. Recently, Hsp90 was shown to be overexpressed in cancer cells, with several oncoproteins identified as its client proteins [3]. Due to its roles in supporting the malignant transformation of tumor cells, Hsp90 has emerged as a promising target for cancer therapy [4].

Hsp90α, an isoform of the Hsp90 family, can also be secreted to the extracellular space [5]. Extracellular Hsp90α was first identified as a tumor specific antigen in fibrosarcoma [6]. Then the expression of Hsp90α on cell surface was also found in lung cancer, melanoma, and lymphoma cells [5]. Recent studies indicate that the secretion of Hsp90α is elevated in malignant tumor cells and is associated with tumor invasiveness and metastasis [5,7]. In addition, extracellular Hsp90α has also been identified in neurons, dermal fibroblasts, macrophages and epithelial cells, and participates in neuronal migration, wound healing and viral and bacteria infections [5].

Angiogenesis is defined as the sprouting of new blood vessels from pre-existing capillaries via endothelial cell proliferation and migration [8]. It is not only imperative for physiological processes such as wound healing, but also contributes to numerous malignant, ischemic, infectious and immune disorders [9]. However, the regulatory mechanisms of Hsp90α secretion from endothelial cells and the relationship of secreted Hsp90α with angiogenesis remain unclear. In the present study, the secretion and function of extracellular Hsp90α during angiogenesis were investigated. We find that the secretion of Hsp90α but not Hsp90β is induced in endothelial cells by angiogenic factors and extracellular matrix (ECM) proteins. Secreted Hsp90α promotes endothelial cell migration and angiogenesis during wound healing in an ECM dependent manner, which provides evidence for the potential application of Hsp90α as a stimulator for wound regeneration, and alternatively, as a target for other angiogenesis-related diseases.

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2. Materials and methods

2.1. Cell lines

HMEC is a human dermal microvascular endothelial cell line (HDMEC, Sciencell) transfected with SV40 large T antigen and cultured in DMEM with 10% fetal bovine serum (FBS) [10].

2.2. Antibodies and growth factors

Anti-Hsp90α antibody (9D2) was from Stressgen Bioreagents (Victoria, Canada). Anti-Hsp90β antibody (Hsp90β mAb) (H90–10) was from Abcam (Cambridge, UK). Anti-Actin antibody (I-19) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD31 antibody (PECAM-1, T709) was from Bioworld Technology (Minneapolis, MN). Monoclonal antibody against Hsp90α (Hsp90α mAb) for endothelial cell migration, tube formation in vitro and wound healing assay in vivo was prepared by our lab [7].

Basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB) and stromal derived factor-1 (SDF-1) were from PeproTech (Rocky Hill, NJ, USA).

2.3. Confocal

HMECs (1×10^5/well) were plated on coverslips coated with polylysine (100 μg/ml), gelatin (200 μg/ml) or fibronectin (10 μg/ml). The quiescent status of the cells was induced by serum starvation, while the activated status was maintained by culturing the cell in DMEM plus 10% FBS. The cultured cells on coverslips were washed by PBS, fixed in 4% paraformaldehyde and blocked by 5% normal goat serum without permeabilization. Then the cells were incubated with anti-Hsp90α or anti-Hsp90β antibody and TRITC-conjugated secondary antibodies. After the incubation, Tween 20 (0.5%) was used to permeabilize the cells, which is mild and gave large enough pores for antibodies or probes penetration but without dissolving the plasma membrane. Actin filaments were thus stained by FITC-phalloidin (Sigma) and nuclei were stained by DAPI. Confocal imaging was performed by a Nikon A1 confocal microscope.

2.4. Western blotting

HMECs were cultured in serum-free media with the indicated treatment for 8 h. The conditioned media (CM) was then collected and concentrated by 10 folds with Microcon YM-10 Centrifugal Filter Units (Millipore, Billerica, MA) at 4 °C. The cells were lysed using buffer containing 50 mM Tris, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, pH 7.5 and protease inhibitors. CM and cell extract samples were applied to SDS–PAGE, electro-transferred onto PVDF membranes, incubated with specific primary and secondary antibodies, and revealed by chemiluminescence (SuperSignal West Pico Substrate, Pierce).

2.5. Vector construction and recombinant proteins preparation

The cDNA encoding the entire open reading frames of human Hsp90α and Hsp90β were amplified from a human liver cDNA library. The amplified sequences were subcloned into pQE80L with His epitope at the N-terminus to generate recombinant Hsp90α (rHsp90α) and Hsp90β (rHsp90β).

rHsp90α was expressed in a soluble form by *E. coli*. The protein was purified by Ni–NTA Agarose (Qiagen, Germany). The purified rHsp90α was dialyzed, quantified, and stored in PBS at −70 °C after being snap frozen by liquid nitrogen [11]. rHsp90β was prepared using the same procedure.

2.6. Migration assay

The in vitro cell migration assay was performed using 6.5-mm Millicell chambers (8 μm pore size, Millipore) with or without Matrigel (50 μg/well) (Vigorous, Beijing, China) coated on the upper side of the filter membranes. 5×10^4 HMECs were inoculated into the Millicell and 0.2% FBS was added in the lower chambers as a chemoattractant. The cells were allowed to migrate for 4 h through the non-coated chambers and 24 h through the Matrigel-coated chambers. Cells at the lower surface of the filter membranes were stained with Hematoxylin & Eosin and five random fields were selected for counting per chamber. The images were captured by a CCD camera (Olympus DP71). Experiments were performed in triplicate and repeated twice.

2.7. Tubule formation assay in vitro

This assay was conducted as previously described [12]. Briefly, 24-well plates were coated with 125 μl/well of Matrigel. HMECs (1×10^5 cells/well) were seeded and cultured for 12 h in DMEM plus 0.2% FBS with the indicated treatment. The tubule was visualized by an Olympus IX71 microscope and images were captured by a CCD camera (Olympus DP71). The tubule length was calculated in three random fields per well using Image-Pro Plus. Experiments were performed in triplicate and repeated twice.

2.8. Scratch assay in vitro

HMECs were plated on the gelatin-coated six-well plate to form a confluent monolayer. A "scratch" was created by scraping the cell monolayer in a straight line with a p200 pipet tip. The cells were washed and cultured in DMEM plus 2% FBS. The images of the "scratch" were acquired after 8, 16, 24, and 32 h incubation of the cells with the indicated treatment. The images were captured by a CCD camera (Olympus DP71). Experiments were performed in triplicate and repeated twice.

2.9. Wound healing model in vivo

This assay was performed as previously described [13]. Briefly, nude mice were anaesthetized with an intraperitoneal (i.p.) injection of urethane (1.5 g/kg). Full-thickness excision wounds (7×7 mm) were made on their dorsal skin. The wounds were covered by 50 μl carboxymethylcellulose (5%) containing Hsp90 recombinant proteins or antibodies (50 μg) and then dressed by the self-adhesive elastic bandage. Hsp90 recombinant proteins or antibodies were applied to the wounds daily from the day of skin excision. The open wound area was determined by vernier calliper every other day. The mice were sacrificed 5 or 9 days post-wounding and the wound areas were collected for the further analyses.

2.10. Determination of angiogenesis in wound

Blood vessel density was determined by the Immuno-fluorescence assay. Paraffin-embedded wound tissues were processed into 5 μm thick sections. Sections were re-hydrated and blocked with 5% normal goat serum, then incubated with rabbit anti-CD31 antibody (1:200, Bioworld) and TRITC-conjugated anti-rabbit IgG. Nuclei were stained with DAPI. Sections were analyzed by a Nikon A1 confocal microscope.

2.11. Immunohistochemistry (IHC) detection of extracellular Hsp90α in vivo

Sections of wounded tissues from the wound healing assay that were treated with control IgG, Hsp90α mAbs or Hsp90β mAbs were

re-hydrated, blocked, and incubated with HRP-conjugated anti-mouse IgG antibody, which recognizes the administered mouse monoclonal antibodies and indicates the distribution of extracellular Hsp90α in vivo. DAB was used as the chromogen and Hematoxylin was used for counterstaining. The images were captured by a CCD camera (Olympus DP71).

2.12. Statistical analyses

Student *t*-tests were used to calculate *P*-values. (**): $P < 0.01$, (*): $P < 0.05$. Error bars: SD.

3. Results

3.1. The secretion of Hsp90α is increased in activated endothelial cells

The process of angiogenesis begins with the activation of endothelial cells from pre-existing blood vessels [8]. To investigate the relationship of extracellular Hsp90α with angiogenesis, the secretion of Hsp90α from quiescent and activated HMECs was examined by detecting cell surface Hsp90α expression using Immuno-fluorescence staining. On the surface of quiescent HMECs, Hsp90α staining was very weak and displayed a dispersed distribution pattern (Fig. 1A, upper). Strikingly, on the surface of activated HMECs, significantly higher amounts of Hsp90α were detected and mainly localized on the leading edge of the cells (Fig. 1A, lower). Meanwhile, the secretion of Hsp90β was also assessed using the same method. Only small amounts of Hsp90β were detected on the surface of both quiescent and activated HMECs (Supplementary Fig. S1), indicating that the secretion of Hsp90α, but not Hsp90β, is specifically elevated in activated endothelial cells.

Several growth factors such as bFGF, VEGF, PDGF-BB as well as chemokines such as SDF-1 have been identified as positive regulators of angiogenesis [8,14]. We next investigated Hsp90α and Hsp90β secretion from HMECs following treatment with these angiogenic factors. The results showed that the secretion of Hsp90α from HMECs was promoted by the stimulation of bFGF, VEGF, PDGF-BB and SDF-1 (Fig. 1B and C). In contrast, Hsp90β could hardly be detected and exhibited no change following treatment with these angiogenic factors (Fig. 1B and C). These results consistently demonstrate that the secretion of Hsp90α is highly elevated in the activated endothelial cells.

3.2. ECM proteins induce the secretion of Hsp90α from endothelial cells

ECM proteins are also involved in the regulation of angiogenesis [15]. Thus the effects of ECM proteins on Hsp90α secretion from HMECs were explored. It was found that when compared with polylysine (the most commonly used attachment factor that does not stimulate the biological activity of cells), both gelatin and fibronectin, which are the major components of the ECM [15], were able to stimulate the secretion of Hsp90α and induce its localization on the leading edge of HMECs (Fig. 2A).

To further confirm this result, the amounts of secreted Hsp90α in conditioned media (CM) from HMECs grown on polylysine, gelatin or fibronectin were compared by Western blotting. As expected, compared with the polylysine treatment, Hsp90α was significantly increased in the CM from cells treated with gelatin and

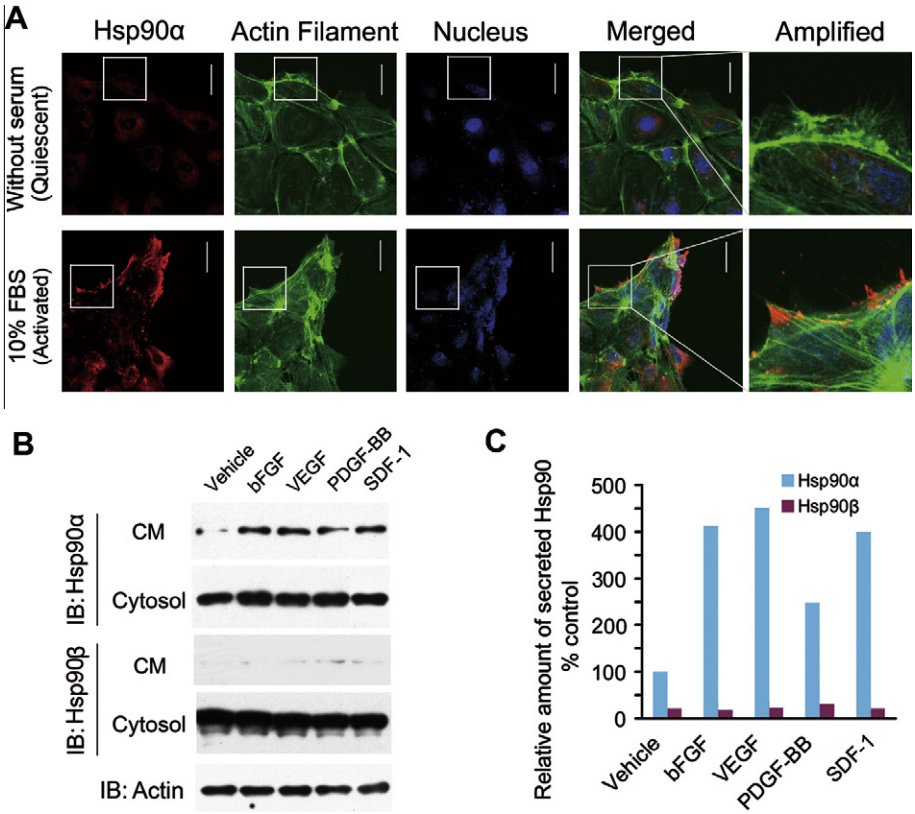

Fig. 1. Angiogenic factors stimulate the secretion of Hsp90α from endothelial cells. (A) The level and distribution of Hsp90α on the surface of quiescent or activated HMECs grown on gelatin were detected by Immuno-fluorescence. Scale bar: 20 μm. (B) The amounts of Hsp90α or Hsp90β in conditioned media (CM) of HMECs treated with angiogenic factors were detected by Western blotting. HMECs grown on gelatin at 80% confluence were treated with serum-free media supplemented with bFGF, VEGF, PDGF-BB, or SDF-1 (20 ng/ml). After 8 h, the CM was collected and examined. (C) Densitometry quantification of western blots shown in panel B. The intensity of the bands was quantified by Gel-Pro Analyzer software.

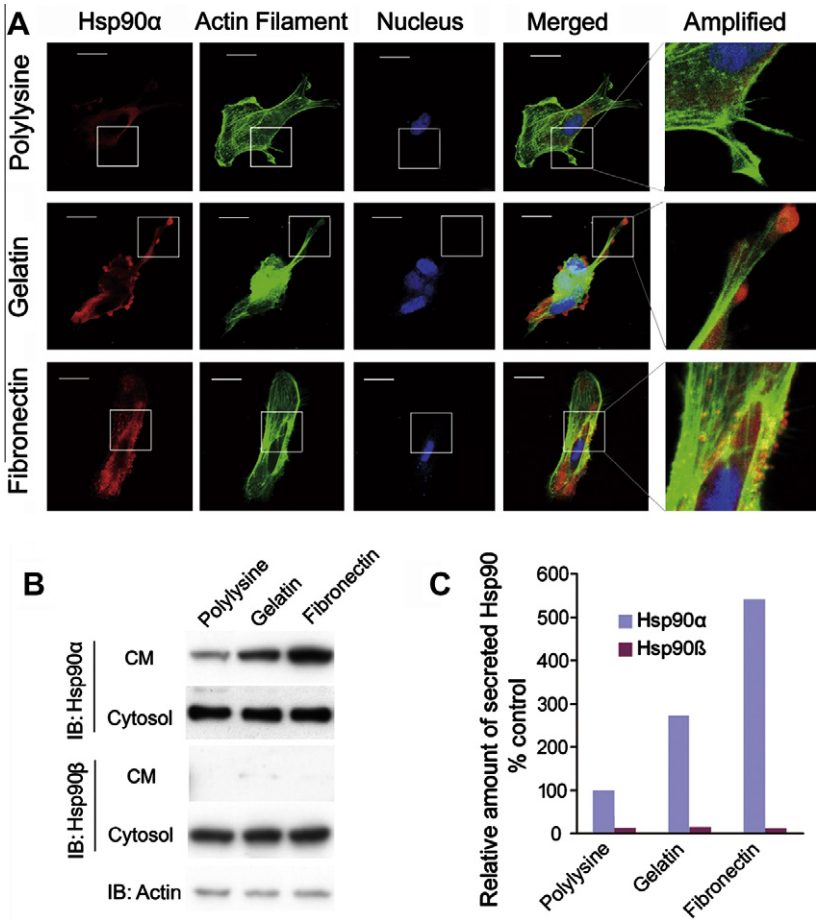

Fig. 2. ECM proteins induce the secretion of Hsp90α from endothelial cells. (A) The level and distribution of Hsp90α on the surface of HMECs grown on polylysine, gelatin, or fibronectin. After 12 h culture in DMEM plus 2% FBS at 37 °C, cell surface Hsp90α was detected by Immuno-fluorescence without the permeabilization of the cells. Scale bar: 10 μm. (B) The amounts of secreted Hsp90α or Hsp90β in the CM of HMECs grown on different ECM proteins were detected by Western blotting. HMECs plated on coverslips coated with polylysine, gelatin or fibronectin at 80% confluence were treated with serum-free media containing 20 ng/ml VEGF for 8 h. The CM was collected and examined by Western blotting. (C) Densitometry quantification of western blots shown in panel B. The intensity of the bands was quantified by Gel-Pro Analyzer software.

fibronectin (Fig. 2B and C). In contrast, Hsp90β showed no obvious secretion following treatment with ECM proteins (Fig. 2B and C). Based on these results, we conclude that ECM proteins, such as gelatin and fibronectin can induce the secretion of Hsp90α from endothelial cells.

3.3. Extracellular Hsp90α promotes endothelial cell migration in an ECM dependent manner

As we demonstrated the secretion of Hsp90α from activated endothelial cells, we asked whether extracellular Hsp90α would be involved in the modulation of angiogenesis. The in vitro Millicell-based cell migration assay was first employed to examine the effect of Hsp90α on the angiogenic activities of endothelial cells. Intriguingly, recombinant Hsp90α (rHsp90α) exhibited no effect on the migration of HMECs across non-coated Millicell chambers (Fig. 3A and Supplementary Fig. S2A), but significantly promoted HMECs migration across the Matrigel-coated chambers (Fig. 3B and Supplementary Fig. S2B). This result indicates that the pro-angiogenic function of Hsp90α is ECM dependent.

To clarify this issue more specifically, we carried out the tubule formation and "scratch" assays in the presence of ECM proteins. As shown in Fig. 3C and Supplementary Fig. S2C, rHsp90α significantly promoted the tubule formation of HMECs on Matrigel, whereas monoclonal antibodies (mAbs) against Hsp90α showed inhibitory effects by blocking the endogenously secreted Hsp90α. Similar results were obtained in the "scratch" assay, which was performed using the HMECs monolayers grown on gelatin (Fig. 3D and Supplementary Fig. S2D). On the other hand, Hsp90β and its neutralizing antibodies showed no effect on both endothelial cell migration and tubule formation (Fig. 3B–D). Taken together, these results demonstrate that extracellular Hsp90α can promote the angiogenic activity of endothelial cells in an ECM dependent manner.

3.4. Extracellular Hsp90α promotes angiogenesis and localizes on the neovasculature in the granulation tissues of wounded mouse skin

Having demonstrated that extracellular Hsp90α promotes the angiogenic activity of endothelial cells in vitro, we then investigated the function of extracellular Hsp90α in angiogenesis using a mouse skin wound healing model in vivo. Wound healing is the physiological process in which skin (or another organ) repairs itself after injury [16]. Angiogenesis is an imperative step for wound healing [17]. To determine the role of Hsp90 in wound healing, recombinant Hsp90 proteins or Hsp90 neutralizing antibodies were directly applied to wounded mouse skin. The wound healing progress was monitored. We found that rHsp90α but not rHsp90β accelerated the process of wound healing, whereas Hsp90α mAbs retarded this process (Fig. 4A and Supplementary Fig. S3A).

Angiogenesis occurs concurrently with the formation of granulation tissue in the wound healing process [17]. Thus blood vessel densities (BVDs) in wounded areas collected 5 days post-wounding, which mainly consist of granulation tissues, were assessed

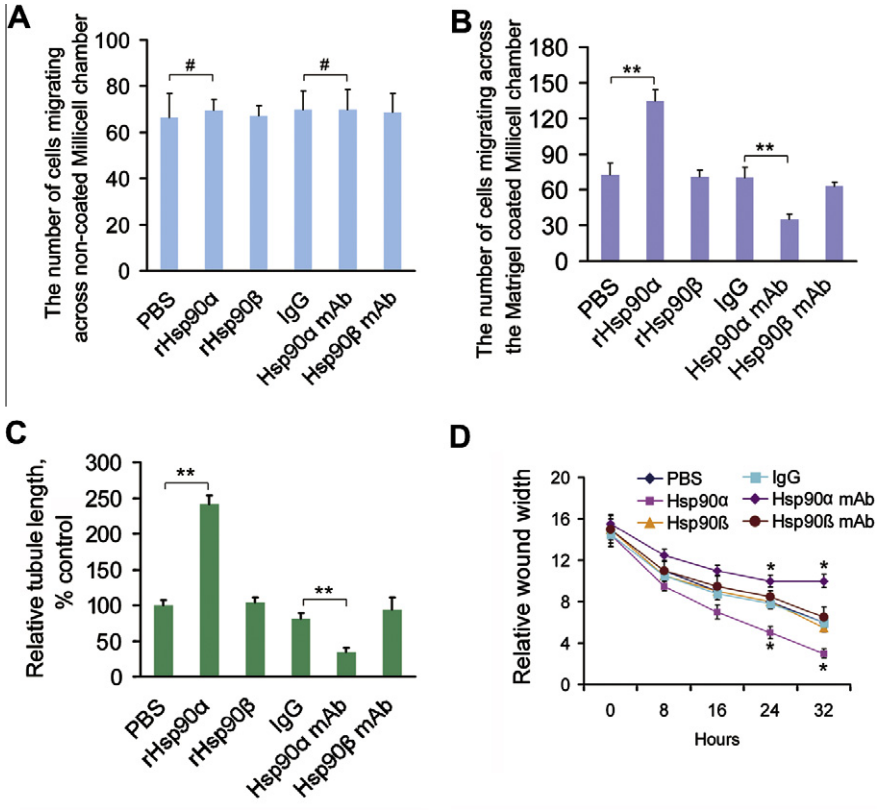

Fig. 3. Hsp90α promotes the angiogenic activities of endothelial cells in an ECM dependent manner. (A-B) The effects of Hsp90 recombinant proteins or neutralizing antibodies (5 μg/ml) on endothelial cell migration through non-coated (A) or Matrigel-coated (B) Millicell chambers. The error bars represent SD ($n = 3$). P value: Student's t-test. *, $P < 0.05$; **, $P < 0.01$; #, $P > 0.05$. (C) The tubule formation of HMECs treated with Hsp90 recombinant proteins or antibodies (5 μg/ml) was examined by Matrigel model. Error bars: SD ($n = 3$). P value: Student's t-test. *, $P < 0.05$; **, $P < 0.01$. (D) The effects of Hsp90 recombinant proteins or antibodies (5 μg/ml) on cell migration were examined by an in vitro "scratch" assay. Error bars: SD ($n = 3$). P value: Student's t-test. *, $P < 0.05$; **, $P < 0.01$.

by the immuno-fluorescence staining of CD31. The results showed that the BVDs were significantly increased in wounds treated by rHsp90α, whereas were reduced in the Hsp90α mAbs treated wounds (Fig. 4B and Supplementary Fig. S3B). This is consistent with the effects of Hsp90α and its neutralizing antibodies on endothelial cell migration and tube formation in vitro (Fig. 3B–D). Extracellular Hsp90α is thus demonstrated to promote angiogenesis both in vitro and in vivo.

Tissue sections of wounds treated with control IgG, Hsp90α mAbs or Hsp90β mAbs in the above assay were further analyzed by IHC. Since the administered Hsp90α mAbs or Hsp90β mAbs could co-localize with the endogenously secreted Hsp90α or Hsp90β in these tissues, the IHC staining by HRP-conjugated anti-mouse IgG would represent the distribution of the endogenous secreted Hsp90 proteins. The results showed that only in granulation tissues treated with Hsp90α mAbs, the blood vessels were positively stained (Fig. 4C), indicating the localization of extracellular Hsp90α on the neovasculature of wounded tissues but not normal tissues. This result provides direct evidence for the association of extracellular Hsp90α with neovasculature, supporting elevated secretion of Hsp90α from activated endothelial cells during wound healing in vivo.

4. Discussion

4.1. The localization of Hsp90α on the leading edge of activated endothelial cells

The localization of Hsp90α on the cell surface has long been observed [6], but its regulatory mechanism and function remain largely unknown. Cheng et al. reported that TGFα could stimulate rapid and robust membrane relocation and cell surface clustering of Hsp90α in keratinocytes [18]. Here we find that angiogenic factors bFGF, VEGF, PDGF-BB and SDF-1, as well as ECM proteins fibronectin and gelatin stimulate Hsp90α secretion and localization on the leading edge of endothelial cells (Figs. 1 and 2A). Together, these results suggest a possible association of Hsp90α with actin cytoskeletal dynamics during cell migration. This association, as indicated here and by Cheng et al., appears to be independent of cell type and of which, the regulatory mechanisms merit further investigation.

4.2. Extracellular Hsp90α but not Hsp90β associates with angiogenic endothelial cells

Hsp90α and Hsp90β are closely related isoforms of Hsp90 [19]. The genes of these two isoforms appear to be differentially regulated during the development or under stress conditions, whereas the proteins share similar functions in chaperoning their client proteins in the cytosol [20]. However, in terms of the extracellular forms, secreted Hsp90α has been well-demonstrated [7], while the secretion of Hsp90β remains controversial [21].

Here we observed that only a small amount of Hsp90β could be detected on the cell surface and in the CM of HMECs (Fig. 1 and Supplementary Fig. S1), and neither angiogenic factors nor ECM could induce Hsp90β secretion (Figs. 1 and 2) or relocation. Moreover, both rHsp90β and Hsp90β mAbs had no effect on angiogenesis in vitro and in vivo (Figs. 3 and 4). Collectively, these results demonstrate that Hsp90α, but not Hsp90β, is specifically elevated and involved in angiogenesis.

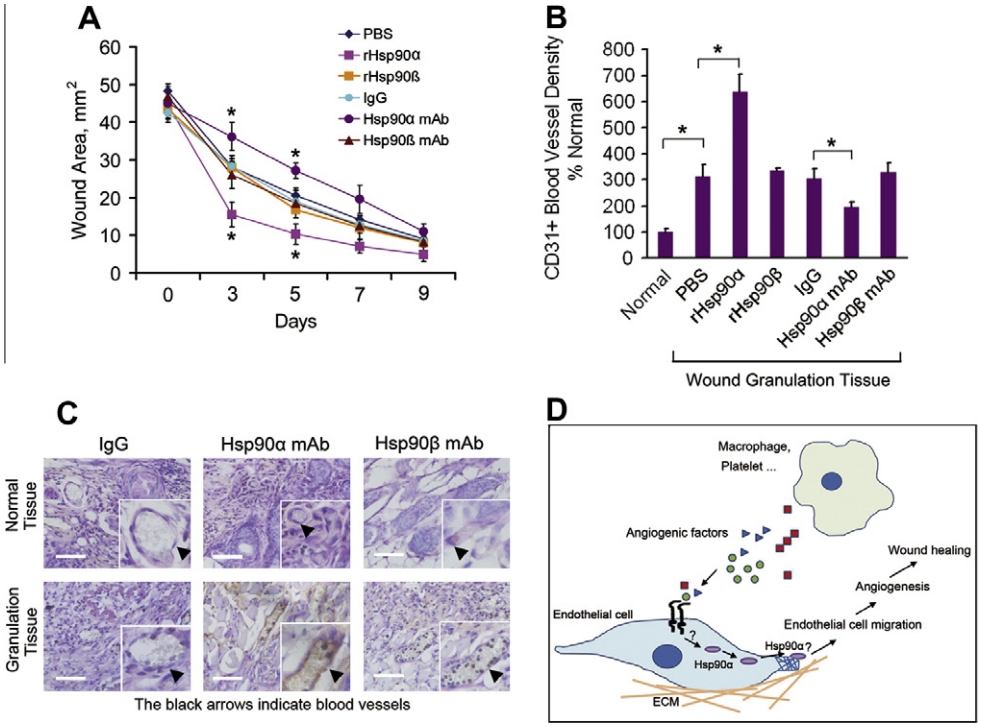

Fig. 4. Hsp90α localizes on the neovasculature and promotes angiogenesis in granulation tissues of wounded skin. (A) The open wound areas of wounded skin treated with Hsp90 recombinant proteins or antibodies (50 μg/day) were monitored and compared. Error bars: SD ($n = 6$). P value: Student's t-test. *, $P < 0.05$; **, $P < 0.01$. (B) The blood vessels in granulation tissues were detected by Immuno-fluorescence using the anti-CD31 antibody. The blood vessel density (BVD) was quantified by NIS-Elements C, the software of Nikon A1 confocal. Error bars: SD ($n = 6$). P value: Student's t-test. *, $P < 0.05$; **, $P < 0.01$. (C) The localization of the administered control mouse IgG, Hsp90α mAbs, or Hsp90β mAbs in granulation tissues was examined by IHC using HRP-conjugated anti-Mouse IgG antibody. Scale bar: 50 μm. (D) A schematic summary of the regulatory mechanism of Hsp90α secretion from activated endothelial cells and its pro-angiogenic function in wound healing.

4.3. The dependency of pro-angiogenic activity of extracellular Hsp90α on ECM proteins

In the study of the pro-angiogenic activity of extracellular Hsp90α in vitro, we found that rHsp90α could promote the migration of HMECs across Matrigel-coated, but not non-coated Millicell chambers (Fig. 3A and B), indicating that the pro-angiogenic activity of extracellular Hsp90α is ECM dependent and may be related to the interaction or proteolysis of matrix proteins. Coincidently, Eustace et al. reported that extracellular Hsp90α could interact with and promote the activity of matrix metalloproteinase-2 (MMP-2) in tumor cells [21]. MMP-2 is a key protease involved in the ECM degradation and is critical for both angiogenesis and tumor invasiveness [22]. Therefore, we propose that the pro-angiogenic activity of extracellular Hsp90α may be attributed to its chaperoning functions on ECM proteases, such as MMP-2 and resulting effects on ECM proteolysis.

4.4. The molecular mechanism of Hsp90α-regulated angiogenesis in wound healing

Our study provides new insights for the role of extracellular Hsp90α in regulating angiogenesis during wound healing. In summary, the molecular mechanism of Hsp90α-regulated angiogenesis in wound healing is proposed as follows. Upon tissue injury, platelets and macrophages, the cells responsible for the clotting cascade and clearance of bacteria and damaged tissues, are recruited to the wound area [17]. These cells secrete a number of angiogenic factors such as bFGF, VEGF, PDGF and SDF-1 [23], which subsequently stimulate the secretion of Hsp90α from the activated endothelial cells. Secreted Hsp90α localizes on the leading edge of migrating endothelial cells and chaperones proteolytic enzymes capable of degrading ECM proteins within the wound area, thus facilitating new blood vessel sprouting and wound remodeling (Fig. 4D).

Additionally, since angiogenesis is not only essential for wound healing, but also contributes to the pathogenesis of several diseases, our study also provides a clue for the application of Hsp90α as a target for other angiogenesis-related disorders, which is a prospect that certainly merits further investigation.

5. Conclusions

Here we demonstrate that Hsp90α, but not Hsp90β, can be secreted by endothelial cells following stimulation with angiogenic cytokines, chemokines, and ECM proteins. Secreted Hsp90α localizes on the leading edge of migrating endothelial cells and facilitates angiogenesis in wound healing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.046.

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