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Crucial role of HSP90 in the Akt-dependent promotion of angiogenic-like effect of glucose-regulated protein94 (Grp94)-IgG complexes

Elisa Tramentozzi [a], Elena Tibaldi [b], Anna Maria Brunati [b], Andrea Pagetta [a], Paola Finotti [a, *]

[a] *Department of Pharmacology and Anesthesiology, University of Padova, Largo E. Meneghetti, Padova, Italy*
[b] *Department of Biochemistry, University of Padova, Viale G. Colombo, Padova, Italy*

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Abstract

Previous observations showed that complexes of glucose-regulated protein94 (Grp94) with human IgG, both those isolated from plasma of diabetic subjects and complexes formed *in vitro*, displayed cytokine-like effects on human umbilical vein endothelial cells (HUVECs), including angiogenic-like transformation capacity that predicted an increased risk of vascular damage. The aim of the present work was to find an effective inhibitor of the angiogenic-like effect of Grp94-IgG complexes. Because this effect is mediated by an increased expression of matrix metalloprotease-9 (MMP-9), we tested the selective MMP-9 inhibitor, the cyclic decapeptide CTT (CTTHWGFTLC) at 5, 10 and 20 μM. CCT failed to inhibit any morphological alteration induced by Grp94-IgG on HUVECs, on its own displaying a paradoxical angiogenic-like activity. We identified the phosphatidylinositol 3-kinase (PI3K)/Akt pathway as the specific target activated by both Grp94-IgG and CTT for sustaining the angiogenic-like transformation of HUVECs. Functioning of the PI3K/Akt pathway was crucially dependent on functional heat-shock protein (HSP)90, and both Grp94-IgG and CTT caused and increased expression of HSP90, promoting its localization to podosomes. CTT appeared to enhance the angiogenic-like effect of Grp94-IgG by increasing the rate of secretion of both HSP90 and MMP-9. By preventing the chaperoning capacity of HSP90 with the inhibitor purine-scaffold (PU)-H71 that blocked the ATP-binding site on HSP90, it was possible to inhibit the expression of Akt and secretion of HSP90 and MMP-9 induced by Grp94-IgG, thus completely reversing the angiogenic pattern. Results reveal a fundamental role of HSP90 in the PI3K/Akt pathway-mediated angiogenic-like effect of Grp94-IgG, also questioning the capacity of CTT to serve as an effective inhibitor of the angiogenic effect.

Keywords: endothelial cells • angiogenic proteins • signal transduction pathways • enzyme inhibitors • heat-shock proteins

Introduction

The role of matrix metalloproteases (MMPs) in remodelling the extracellular matrix and inducing cell migration in both physiological and pathological conditions has been reviewed extensively [1, 2]. In particular, the increased expression and activity of gelatinases MMP-2 and MMP-9 have been demonstrated to contribute to the pathogenesis of several inflammatory diseases and tumours [1, 3–5] Besides the well-known proteolytic degradation of type IV collagen, MMPs might induce the migration of inflammatory and tumour cells by forming new capillary vessels, concurring in the development of pathological angiogenesis [1, 2, 6]. Of interest is the finding that growth factors and cytokines that stimulate the expression and cellular secretion of heat-shock proteins (HSPs) also stimulate the expression of MMPs, thus enhancing the inflammatory response and promoting its spreading by autocrine–paracrine mechanisms [7–9].

We recently demonstrated that in the plasma of type 1 diabetic subjects the antibody fraction that was stably complexed with the glucose-regulated protein94 (Grp94) stimulated the angiogenic differentiation of human umbilical vein endothelial cells (HUVECs) by a mechanism that involved the increased expression of both HSP90 and HSP70, the latter resulting closely associated with a cell membrane-bound inactive species of MMP-9 [10]. Our results supported the conclusion that circulating Grp94-IgG complexes might be a marker of an increased angiogenic risk sustained by HSP-chaperoned, proteolytically inactive forms of MMP-9. This

*Correspondence to: Paola FINOTTI,
Department of Pharmacology and Anesthesiology,
L.go E. Meneghetti, 2, 35131 Padova, Italy.
Tel.: +39 049 8275088
Fax: +39 049 8275093
E-mail: paola.finotti@unipd.it

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conclusion was in accord with other reports demonstrating that proteolytic activity of MMPs only partially and indirectly is involved in the process of angiogenesis [1]. In order to investigate the molecular mechanism by which Grp94-IgG complexes might act *in vivo*, we obtained complexes *in vitro* formed with native Grp94 and human IgG that served as effective substitute of plasma-purified complexes [11]. Effects of Grp94-IgG complexes appeared to be mediated by an increased expression of the MMP-9 pro-form in turn sustained by the activation of the mitogen-activated extracellular kinase1/2 (MEK-ERK1/2) pathway [1, 12]. However, the specific inhibition of the MEK-ERK1/2 pathway failed to affect the MMP-9 expression as well as the angiogenic transformation of HUVECs [1, 11, 12]. With these results in mind, and in light of the implications that inhibition of pathological angiogenesis promoted by a stable rise of angiogenic factors might have for any effective anti-inflammatory and anti-tumour therapy [5, 6], it was of interest to test whether inhibitors of MMPs could antagonize angiogenic-like effect of the Grp94-IgG complexes. Among the several molecules developed as MMP inhibitors, the selective inhibitor of MMP-2 and MMP-9, the cyclic decapeptide CTTHWGFTLC (abbreviated CTT) appeared the best candidate as it was particularly effective in suppressing migration of endothelial cells *in vitro*, and in preventing tumour growth and invasion in mice [13, 14].

Although the molecular mechanism by which CTT displays its effects is not yet clear [1], it is generally accepted that inhibition of MMP-9 enzyme activity *per se* is not sufficient to abolish endothelial cell proliferation and migration [1, 14]. It has been proposed that the hydrophobic nature of CTT might favour its binding to phospholipids of the cell membrane, a condition that would permit CTT to target cell surface-bound gelatinases [15].

The aim of our work was thus to test the inhibitory capacity of CTT on angiogenic-like transformation of HUVECs induced by Grp94-IgG complexes formed *in vitro*. We used these complexes as surrogate of those purified from diabetic plasma, because the latter can neither be obtained in a sufficient amount nor separated in the purification procedures from the bulk of plasma IgG not complexed with Grp94 [10].

Results unexpectedly revealed that CTT not only was completely unable to counteract the effects of Grp94-IgG on HUVECs, but it also displayed similar capacity to stimulate the differentiation process in HUVECs. Inspection of the molecular mechanism involved revealed the importance of HSP90-driven stimulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in sustaining the angiogenic effect of Grp94-IgG complexes and the role that specific HSP90 inhibitors might thus have in blocking the angiogenic transformation.

Materials and methods

Reagents

Endothelial basal medium (EBM), foetal bovine serum (FBS), antibiotics were from Lonza (Lonza, Walkersville, MD, USA); MMP-2/MMP-9 inhibitor CTT and U0126 were from Calbiochem (Merk KGaA, Darmstadt, Germany); LY294002, human pre-immune IgG, gelatine, endothelial cell growth supplement, bovine serum albumin, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Mowiol 40–88 were from Sigma (Sigma Chemicals, St. Louis, MO, USA); anti-human HSP90α/β rabbit polyclonal Abs were from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-human rat HSP90α monoclonal, rabbit HSP70 polyclonal and mouse HSP70 monoclonal Abs were from StressGen (StressGen Biotechnologies, Victoria, B.C., Canada); mouse anti-β actin monoclonal Abs were from Cell Signaling & Neuroscience (Cell Signaling & Neuroscience, St. Louis, MI, USA); anti-MMP-9 mouse monoclonal Abs were from Calbiochem. Anti-Akt and P-Akt Abs were from Santa Cruz Biotechnology Inc. All other reagents were of analytical grade from Sigma. PU-H71 was a generous gift of Dr. G. Chiosis (Memorial Sloan-Kettering Cancer Center, NY, USA).

Grp94-IgG complex

The Grp94-IgG complex was obtained by incubating native Grp94, purified from rat liver microsomal fractions, with human, pre-immmune IgG at the molar ratio of 1:1 for 1 hr at 37°C, and its formation tested as previously specified [11]. The Grp94-IgG complex was used in experiments of cell cultures at the final concentration of 10 ng/ml, because in previous experiments this was proven to be effective in supporting the proliferation and angiogenic transformation of HUVECs [11].

Cell cultures

HUVECs were isolated from freshly collected umbilical veins by collagenase treatment [16]. At least three different fresh cords were employed for any cell preparation. Cells were maintained in EBM supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 10 μg/ml streptomycin, 20 μg/ml endothelial cell growth supplement at 37°C in a humidified 95% air, 5% CO_2 atmosphere, until the cells reached sub-confluence. Cell culture purity was assessed by microscopic examination of the typical cobblestone morphology and by phycoerythrin (PE) mouse anti-human CD31 monoclonal Abs (BD Pharmingen™, San Josè, CA, USA). All experiments were performed with HUVECs at passages 3–5.

Cell proliferation assays

Proliferation was evaluated by means of both the MTT test and cell counting. In the former, HUVECs were seeded in the number of 1.5×10^4 in 96-well plates (200-μl well) in EBM supplemented with 10% FBS, under the condition of atmosphere and temperature specified above. After starvation in serum-free medium for 6–8 hrs, cells were supplemented with a fresh aliquot (200 μl) of serum-free medium, without (control) and with Grp94-IgG (10 ng/ml) and CTT, added both singularly and together, in quadruplicate wells. CTT was dissolved in pure de-ionized water with 1% DMSO and used at the final concentrations of 5, 10 and 20 μM. CTT was added 15 min. prior to the addition of the Grp94-IgG solution, whereas an equivalent volume of solvent was added in wells without CTT. After the incubation time of 18 hrs, 20 μl of MTT (5mg/ml in phosphate-buffered saline [PBS]) were added to each well and left in incubation for 4 hrs. The supernatant was then discarded and 100 μl of isopropanol containing 0.04 M HCl added for solubilization of formazan crystals. Absorbance was

read at 570 and 630 nm and the difference between the two wavelengths taken as the value expressing the proliferation value.

In other experiments, HUVECs (25×10^4/well) were seeded in 12-well (2 ml each) plates and allowed to attach to well plastics for 24 hrs. Cells were then starved in fresh, serum-free medium for 6–8 hrs and after this time, aliquots of both Grp94-IgG complex and CTT solutions added in fresh medium, as specified above for the MTT assay. After incubation at 18 hrs, the medium was collected and stored at –20°C before further analysis, cells washed with PBS, detached from duplicate wells by adding 0.05% trypsin and 0.2% ethylene diamine tetraacetic acid (EDTA), and counted in a haemocytometer. Cell viability was evaluated with the trypan blue dye exclusion method.

In experiments in which inhibitors were tested, these were added to the cell culture 15 min. before the addition of Grp94-IgG complex solution (where specified). The MEK-ERK1/2 inhibitor U0126 was employed at the final concentration of 10 μM, whereas the PI3K/Akt pathway inhibitor LY294002 was used at final concentrations of 25, 20, 15, 10 and 5 μM.

Analysis of cell lysates

After each incubation time, cells were washed with PBS, scraped and centrifuged for 15 min. at 600 × *g*. After removal of the supernatant, cells were lysed in the Laemmli lysis buffer (50 mM Tris-HCl, pH 8.9, 5 mM EDTA, 380 mM glycine, 2% SDS) (whole lysates). Proteins were measured by the micro-BCA assay (Pierce, Rockford, IL, USA), unless otherwise specified. Whole lysates were then analysed by SDS-PAGE on 10% polyacrylamide gel, followed by blotting on a nitrocellulose membrane for measuring ERK1/2 and Akt activity with total and phospho(P)-specific ERK1/2 polyclonal Abs (Cell Signaling & Neuroscience) and with total and P-Akt-specific polyclonal Abs. Immuno-detection was performed with either the enhanced luminol-based chemiluminescent system (Amersham Biosciences, Uppsala, Sweden) or phosphatase alkaline-conjugate, affinity-purified IgG (from Sigma) and biotin conjugate, affinity-purified IgG (Vector Laboratories, Burlingame, CA, USA) coupled with white avidin conjugated to alkaline phosphatase (ABC system). Abs against β actin were used as controls for protein loading.

In separate experiments, cell lysis was also performed in sterile de-ionized water followed by centrifugation at 100,000 × *g* for 90 min. The supernatant was collected (cytosol fraction) and the pellet (membrane fraction) treated with the Laemmli buffer (native lysates). The pellet was then analysed by SDS-PAGE on 4–20% gradient polyacrylamide gel followed by immunoblotting with anti-HSP70, anti-HSP90α/β and anti MMP-9 Abs.

Analysis of media

Media from duplicate wells of control and treated HUVECs were collected, centrifuged for 10 min. at 800 × *g* to remove cell debris, and dialysed overnight at 4°C against high grade pure distilled water. The lyophilized material was re-suspended in 100 μl of sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS) before submitting samples to SDS-PAGE (10% polyacrylamide gel) and Western blotting with anti-MMP-9, anti-HSP90α/β and HSP70 Abs.

Proteolytic activity of media was measured by means of zymogram gel analysis by loading samples on to the polyacrylamide gel (10%) co-polymerized with gelatine (0.8 mg/ml) in the presence of SDS. After repeated washings (15 min. each) with the renaturing solution (2.5% Triton X-100), the gel was incubated overnight at 37°C in a solution of Tris buffer (50 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.4) under slow shaking. The gel was then submitted to staining with Coomassie brilliant blue, followed by de-staining with a 5% methanol and 7.5% acetic acid solution (in de-ionized water) until clear bands appeared against the blue background.

Immunofluorescence microscopic analysis

Cells (15×10^4/well) were seeded in 24-well (1 ml each) plates with detachable glass bottom. After an 8-h starvation, a serum-free aliquot of fresh medium was added without (control) and with Grp94-IgG complexes (10 ng/ml) and 10 μM CTT, added both singularly and together. CTT was added to the cell culture 15 min. before the addition of the Grp94-IgG complex solution. After 16 hrs incubation, glass bottom of each well was gently detached and cells fixed with 4% formaldehyde in PBS for 15 min., washed three times, and treated with 0.1% Triton X-100 in PBS at room temperature for 10 min. After two further washings with PBS, cells were incubated for 30 min. with blocking buffer (PBS containing 1% bovine serum albumin), washed twice with PBS and incubated for 2 hrs at 37°C with both phalloidin (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA) in PBS (at a 1:100, v:v ratio), to evaluate overall cell morphology and the actin cytoskeleton, and rabbit anti-human HSP90α/β and HSP70 Abs (1:100, w:v ratio). Alexa Fluor 488 goat anti-rabbit IgG (1:350, v:v ratio, Molecular Probes) were then added to detect the fluorescent signal of both HSP90 and HSP70. After incubation with specific Abs for 1 hr at room temperature, cells were treated with 21 μg/ml DNase-free RNase in PBS for 10 min. at room temperature. Then, cells were treated with red-fluorescent Propidium Iodide for nuclear and chromosome counterstaining (Molecular Probes), added to Mowiol 40–88 at the final concentration of 0.5 μg/ml.

Statistical analysis

Data were presented as mean ± S.D. unless otherwise stated. GraphPad Prism (GraphPad software, Inc. San Diego, CA, USA) was used for the statistical analysis of data. Comparison between group means was made by applying the two-tailed, unpaired Student's t-test.

A *P*-value <0.05 was considered statistically significant.

Results

CTT inhibits cell growth stimulation but not angiogenic-like transformation of HUVECs induced by Grp94-IgG

We used CTT at concentrations of 5, 10 and 20 μM that comprised the IC_{50} of gelatinase activity *in vitro* (5 μM) and the concentration at which CTT is reported to partially inhibit human endothelial cell migration (20 μM) [13]. We considered these concentrations more physiological than the higher ones (up to 500 μM) used to obtain more effective inhibitory activity by CTT [13] but driving the risk of unspecific effects in experiments *in vitro*.

Grp94-IgG complex (10 ng/ml) stimulated the cell growth by 20% ± 5.44 (mean ± S.E.M., $n = 11$) a value statistically significant with respect to the control ($P = 0.02$, two-tailed Student's

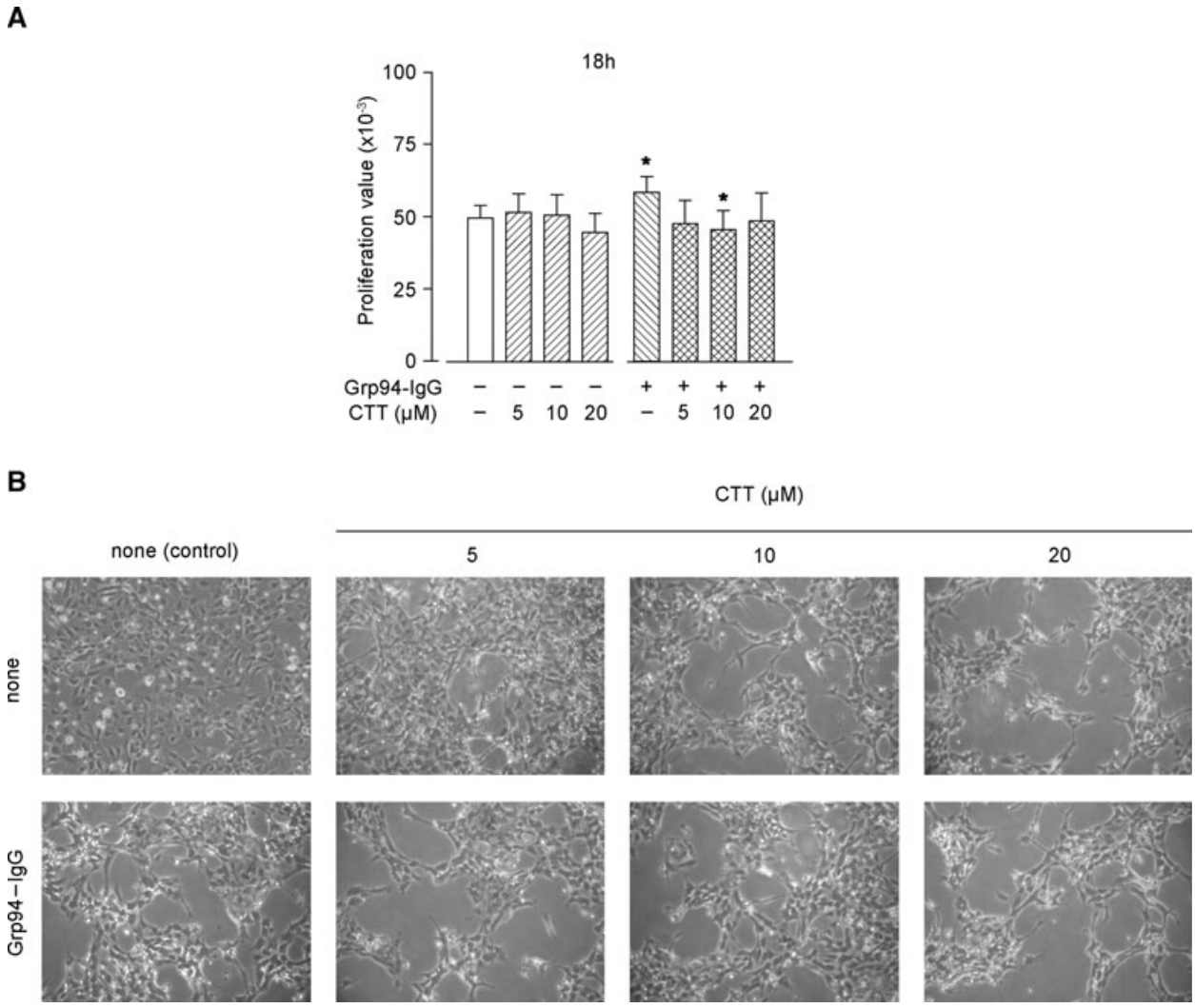

Fig. 1 Effects of CTT on cell growth and morphology of HUVECs in absence and presence of Grp94-IgG complexes. (**A**) Cells were seeded (1.5×10^4) and cultured as specified in 'Materials and methods' in absence (control) and presence of Grp94-IgG complexes (10 ng/ml) added both alone and with CTT at the indicated concentrations. After 18 hrs incubation, cells were treated with MTT (see 'Materials and methods') and the entity of proliferation (on the ordinate axis) expressed as the difference of the absorbance values measured at 570 and 630 nm. (**B**) Cells (25×10^4) were cultured in duplicate wells (2 ml) with both CTT and Grp94-IgG complexes, as above, and after 18 hrs incubation, analysed at the optical microscope. Representative pictures of each condition are shown with original magnification of 10×.

t-test). This effect was abolished by CTT at 5 and even more at 10 μM (mean ± S.E.M. cell growth reduction with respect to the control: 22% ± 6.63, $n = 11$, $P = 0.039$, two-tailed Student's t-test). CTT on its own did not affect at all the proliferation of HUVECs at any of the concentrations used (Fig. 1A).

Microscopic inspection of cell morphology besides confirming the angiogenic-like transformation of HUVECs by Grp94-IgG, unexpectedly showed that CTT was not only unable to antagonize the angiogenic-like transformation of HUVECs by Grp94-IgG but also on its own displayed changes in morphology of HUVECs that mostly at 20 μM overlapped those induced by Grp94-IgG alone (Fig. 1B). Apparently, thus, while inhibiting the proliferative effect of Grp94-IgG on HUVECs, CTT left unaltered the angiogenic-like transformation, a result that suggested a distinct mechanism of action in the activation of either one process by Grp94-IgG.

The MEK-ERK1/2 pathway is dispensable for the angiogenic-like effect of the Grp94-IgG complex

We previously demonstrated that both the stimulation of endothelial cell proliferation and induction of differentiation by Grp94-IgG were

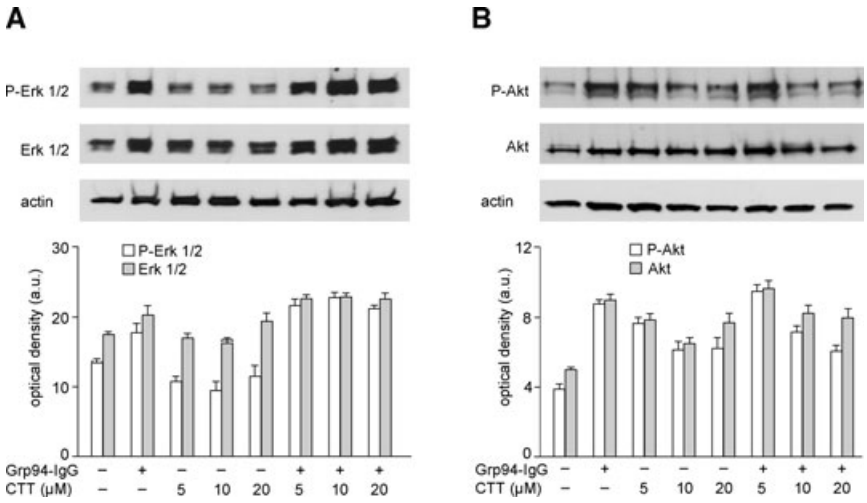

Fig. 2 Effects of CTT on stimulation of MEK-ERK1/2 and Akt pathways induced by Grp94-IgG complexes. Cells (25×10^4) were seeded in duplicate wells (2 ml) in absence (control) and presence of Grp94-IgG complexes (10 ng/ml) added both alone and with CTT at the indicated concentrations, as specified in 'Materials and methods'. After 18 hrs incubation, cells were lysed with the Laemmli buffer added with 7 mM β-mercaptoethanol, and whole lysates analysed in Western blotting for total and phosphorylated forms of ERK1/2 (**A**) and Akt proteins (**B**). Membranes were then probed for β actin for normalization of protein content. A representative Western blotting of five independent analyses is presented for each protein. The height of histograms, shown below the blottings in both (**A**) and (**B**), represents the mean (±S.D.) of densitometric analysis of total and phosphorylated forms of both proteins measured in all experiments ($n = 5$).

mediated by an intense activation of the MEK-ERK1/2 pathway, even higher than that observed with native Grp94 alone [11]. An intense and prolonged activation of ERK1/2 is recognized to underlie the differentiation of HUVECs into tubule-like formations [17, 18]. Because CTT on its own was apparently as active as Grp94-IgG in inducing the differentiation process of endothelial cells, it was of interest to see whether MEK-ERK1/2 pathway was further stimulated by the addition of CTT. After 18 hrs incubation, an intense phosphorylation of ERK1/2 was observed with Grp94-IgG (Fig. 2A), further stimulated by pre-treatment with CTT, irrespective of the concentration, a finding that supported the permissive effect of CTT on the induction of angiogenic-like transformation of HUVECs. The same result however excluded that the MEK-ERK1/2 pathway was directly involved in mediating the inhibition of cell proliferation by CTT. Because CTT alone did not affect at all the phosphorylation of ERK1/2 (the signal was even lower than that of control) (Fig. 2A), the effect of enhancement of ERK1/2 phosphorylation by CTT could be explained by invoking an indirect mechanism of activation by CTT on a pathway, also targeted by Grp94-IgG, other than the MEK-ERK1/2 one.

That the differentiation effect of Grp94-IgG on HUVECs was independent of the MEK/ERK1/2 pathway was confirmed by experiments in which cells were treated with the MEK-ERK1/2 inhibitor U0126 (10 μM, final concentration) before the addition of both Grp94-IgG and CTT. Although U0126 markedly reduced the ERK1/2 phosphorylation in both control and Grp94-IgG-treated cells, with or without CTT, it nevertheless left unchanged the effect of Grp94-IgG on cell differentiation, both in absence and presence of CTT, (data not shown), *i.e.* cell morphology was similar to that observed in absence of U0126. This finding further corroborated the idea that Grp94-IgG sustains the angiogenic-like transformation of HUVECs mainly targeting cellular pathways other than the MEK-ERK1/2 that might be involved secondarily through the cross-talk with closely associated pathways(s) eventually leading to the intense downstream activation of P-ERK1/2 [11].

The PI3K/Akt pathway mediates the angiogenic-like effect of the Grp94-IgG complex

The possibility was then considered that the PI3K/Akt pathway could mediate effects of Grp94-IgG on HUVECs, because this pathway plays a pivotal role in the process of endothelial cell proliferation and differentiation [19]. Both total and phosphorylated forms of Akt of cell lysates were intensely stimulated by Grp94-IgG after 18 hrs incubation (Fig. 2B), a finding that closely resembles what has been observed with some angiogenic promoters (such as VEGF) that affect the Akt activity not only at the level of phosphorylation but also modifying the expression of the protein by a receptor-mediated mechanism of activation of regulatory cellular pathways [20]. CTT was able to antagonize the stimulatory effect of Grp94-IgG at both 10 and 20 μM but not at 5 μM, although inhibition did not restore values of Akt and P-Akt to values of the control (Fig. 2B). At variance with what observed on the ERK1/2 phosphorylation, CTT on its own stimulated the PI3K/Akt pathway by increasing the expression of both Akt and P-Akt, although the overall effect was at any concentration of CTT less intense than that of Grp94-IgG alone. Thus, despite the independent activation of the PI3K/Akt pathway by Grp94-IgG and CTT, the association led to a reduction of the stimulatory effect of Grp94-IgG by CTT, in a concentration-dependent manner.

HSP90 is involved in the activation of the PI3K/Akt pathway by both Grp94-IgG and CTT

It has been reported that intracellular Akt is a client protein of HSP90 that forms complexes in which Akt is stabilized and rendered functionally active [21]. Results of our previous work supported the proposal that the angiogenic effect of Grp94-IgG is

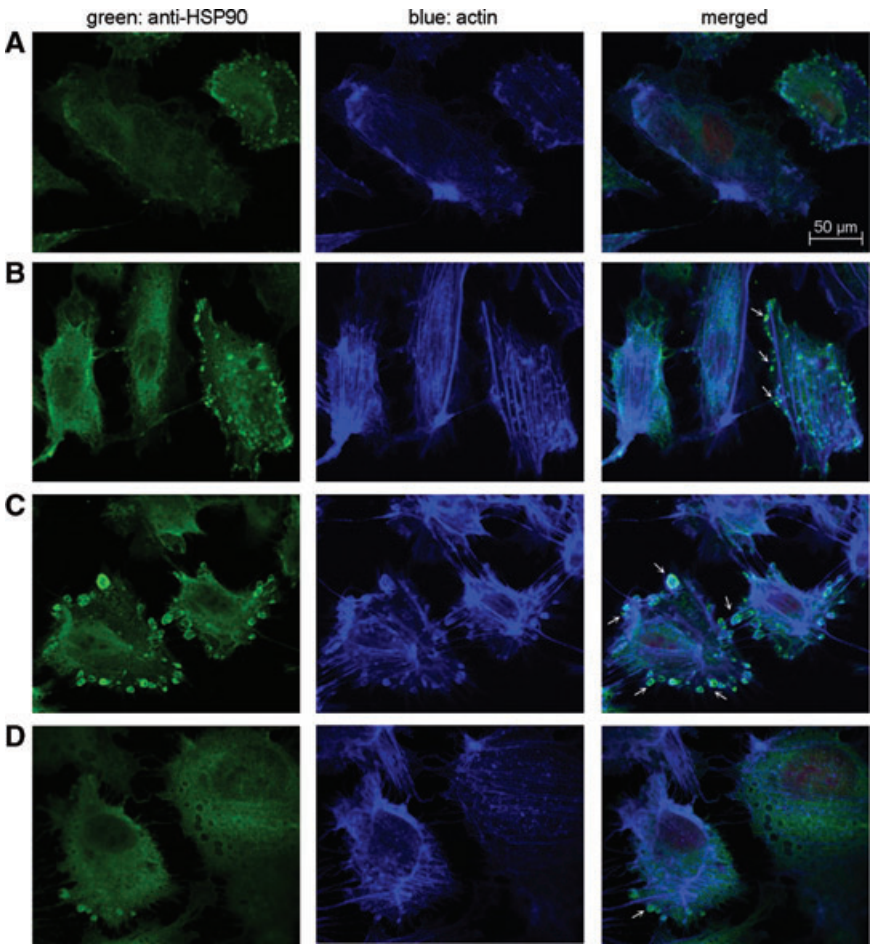

Fig. 3 HSP90 expression is stimulated by individual treatment with Grp94-IgG complexes and CTT. Cells (15×10^4) were cultured in serum-free medium and left either untreated (control, panels **A**) or treated with Grp94-IgG complexes (10 ng/ml) and CTT (10 μM), both alone (panel **B** and **C**, respectively, for Grp94-IgG complexes and CTT) and together (panels **D**), as reported in 'Materials and methods' for immunofluorescence experiments. Individual and merged fluorescence for HSP90 and actin are shown. Co-localization of HSP90 and actin gives rise to a pale blue fluorescence (merged). Numerous podosomes are present in treated cells and contain both actin and HSP90 (arrows in panels **B** and **C**, merged fluorescence).

mediated by autocrine–paracrine mechanisms of activation of HSP90 and HSP70 [11]. Thus, if CTT was able to inhibit the Grp94-IgG dependent activation of the PI3K/Akt pathway by positively targeting the same pathway, one might expect that the HSP90 expression were similarly affected. To investigate this issue, we performed immunofluorescence experiments with both anti-HSP90 and anti-HSP70 Abs on HUVECs treated with Grp94-IgG and CTT at 10 μM, both alone and together. In the former experiment, Grp94-IgG induced the appearance of numerous podosomes in HUVECs in which the HSP90-related fluorescence concentrated mostly in association with actin (Fig. 3, panels b), to testify the intense cytoskeleton modification accompanying proliferation and angiogenic transformation of cells. CTT at 10 μM led to a significant reduction in both the podosomes number and HSP90-dependent fluorescence sustained by Grp94-IgG, although it did not antagonize completely this effect, and HSP90 expression appeared to be still increased with respect to the control (Fig. 3, panels d). CTT alone was as effective as Grp94-IgG in stimulating the podosome formation and in inducing the expression of HSP90 (Fig. 3, panels c), a result that fit both the morphological alteration and stimulation of Akt observed with CTT alone. The results also pointed to the close molecular link between activation of the Akt pathway and increased expression of HSP90 in the cell membrane, as predicted by the function of HSP90 in remodelling the actin cytoskeleton during cell differentiation and migration [22].

At variance with HSP90, the expression of HSP70 in immunofluorescence experiments, although increased with respect to the control after treatment with Grp94-IgG, was neither so intense, nor was it modified at all by CTT (data not shown). Because CTT *per se* did not affect the expression of HSP70 (data not shown), the results supported the possibility that induction of the HSP70 expression during HUVEC differentiation is under the control of pathways, targeted by Grp94-IgG but not by CTT, distinct from the PI3K/Akt.

To confirm results of experiments of immunofluorescence, we also conducted immunoblotting experiments on lysates of HUVECs obtained in native conditions, in which the membrane fraction was separated from cytosol and the expression of HSP90 measured on the pellet for the most part formed with cell membrane. HSP90 was almost absent in cell membranes of control

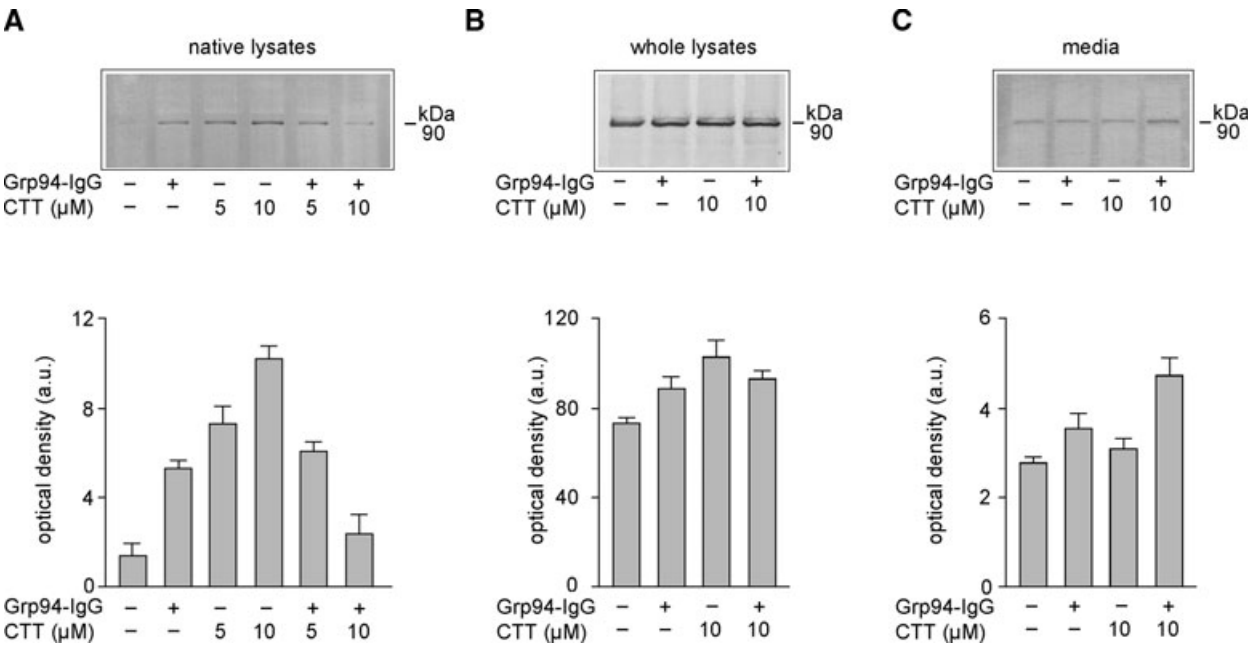

Fig. 4 CTT enhances the Grp94-IgG complex-induced increase in HSP90 secretion from HUVECs. Cells (25×10^4) were cultured as specified in 'Materials and methods' and after a 18 hrs incubation in absence (control) and presence of Grp94-IgG complexes (10 ng/ml) and CTT (10 μM), both alone and together, cells were lysed and Western blotting for HSP90 performed on both the membrane fraction (native lysates) (**A**) and on whole cell lysates (**B**). The same quantity of proteins was loaded in each lane without the addition of β-mercaptoethanol. (**C**) Media were collected from cultured cells before cell lysis (as specified above), lyophilized and re-suspended in sample buffer (without the addition of β-mercaptoethanol) for being analysed in Western blotting with anti-HSP90α/β Abs. Fifteen microlitres were loaded in each lane. A representative Western blotting of four independent analyses is presented in (**A**), (**B**) and (**C**). Below each blotting is the graph with histograms representing the mean (±S.D.) of densitometric analysis of the bands measured in all experiments ($n = 4$).

HUVECs, whereas it was markedly increased with both Grp94-IgG and CTT alone (Fig. 4A). When added together, CTT inhibited the stimulation of HSP90 by Grp94-IgG at 10 μM but not at 5 μM, thus confirming the antagonism seen in immunofluorescence experiments.

Because membrane-bound HSP90, *i.e.* HSP90 associated with cytoskeleton proteins, such as actin, does not give a true information about the whole cell expression of HSP90, mostly confined to the cytoplasm, nor is it an index of the extent of HSP90 secretion, we performed experiments of immunoblotting on whole cell lysates and in media of cells treated with Grp94-IgG in both absence and presence of 10 μM CTT. It was thus apparent that CTT was as able as Grp94-IgG in stimulating the expression of HSP90 in HUVECs, but it was ineffective in reducing the stimulation sustained by Grp94-IgG (Fig. 4B), a finding that was at odds with results of immunofluorescence experiments. Similarly, measurements of HSP90 in media showed that CTT, instead of inhibiting, further increased the stimulation of HSP90 secretion by Grp94-IgG (Fig. 4C). Thus, not only was the expression of HSP90 in HUVECs left unchanged, or even increased by CTT in both absence and presence of Grp94-IgG, but also the antagonism exerted by CTT on the Grp94-IgG dependent stimulation of HSP90 in the cell membrane was only apparent, reflecting instead the capacity of CTT to enhance the effect of Grp94-IgG to stimulate the rate of HSP90 secretion.

CTT does not counteract the effects of Grp94-IgG on the MMP-9 expression and secretion

It has firmly been established that mitogenic stimuli that promote cell proliferation and differentiation also lead to an increased expression of MMP-9 [23], and that MMP-9 expressed by endothelial cells is critical for inducing angiogenesis [6, 12]. However, mechanisms by which MMP-9 might contribute to the development and progression of angiogenesis are still controversial, although it appears to be for most part independent of MMP-9 proteolytic activity [10, 11, 24]. Despite the intensive effort spent in this direction, the signal transduction pathway(s) specifically involved in regulating the expression of MMP-9 following application of various stimuli has not been identified with precision. While the phosphorylation of ERK1/2 appears to be not sufficient for the expression of MMP-9 [12], the role of activated PI3K/Akt pathway in driving stimulation of MMP-9 production and secretion [8, 25] has been considered with much higher relevance. In immunoblotting experiments with monoclonal anti-MMP-9 Abs

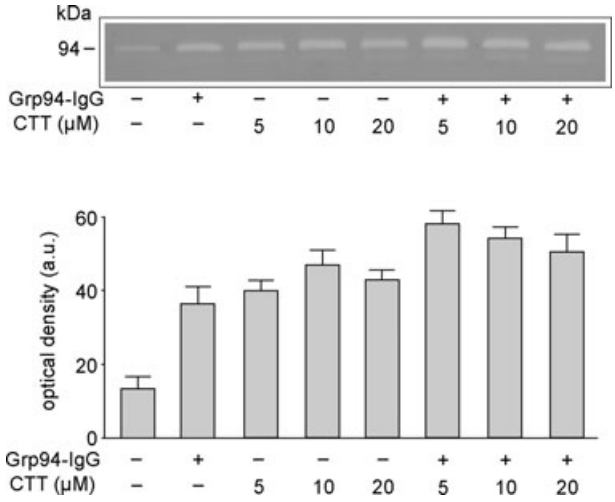

Fig. 5 CTT does not antagonize the Grp94-IgG complex-dependent increase in the MMP-9 secretion. Media collected from cultured cells in absence (control) and presence of Grp94-IgG complexes (10 ng/ml) and CTT, both alone and together, were analysed in gel zymography for gelatinase activity, as specified in 'Materials and methods'. A representative gel zymography of ten independent experiments is reported, showing the digestion band referred to the inactive, 94 kD MMP-9 pro-form, activated experimentally by zymography. Fifteen microlitres were loaded in each lane. Below is the graph with histograms representing the mean (±S.D.) of the optical density (in arbitrary units) of the bands measured in all experiments ($n = 10$).

on whole lysates of HUVECs treated with Grp94-IgG, with and without CTT, we were unable to measure the expression of MMP-9, whereas the inactive (94 kD) form of MMP-9 was detected in gel zymography experiments on culture media, to testify active secretion. Much less intense and of variable intensity was the digestion band referred to the active form of MMP-9 at 92 kD. The MMP-9 pro-form underwent an intense stimulation with both Grp94-IgG and CTT alone (Fig. 5). Again, when tested together, CTT did not reverse at all the stimulation sustained by Grp94-IgG, causing instead a further increase in the area of the digestion band of MMP-9 pro-form that is activated experimentally by zymography. The results are in accord with those reported above, supporting the possibility that the synergic stimulation of the PI3K/Akt pathway by Grp94-IgG and CTT can also mediate the increase in MMP-9 pro-form expression and secretion.

The HSP90 inhibitor PU-H71 inhibits the stimulation of both HSP90 expression and Akt activity induced by Grp94-IgG

Because CTT was devoid of any inhibitory effect on angiogenic-like activity of Grp94-IgG, being in addition on its own a promoter of angiogenesis, and because this effect appeared to be mediated by an HSP90-dependent activation of the Akt pathway, we searched whether an inhibitor of this pathway could effectively antagonize the major Grp94-IgG effect on HUVECs. To this aim, we chosen an inhibitor of the HSP90 function that causes down-regulation of the Akt kinase-dependent pathway instead of the classic inhibitor LY294002 that in our cell cultures reduced cell viability by 40% in control HUVECs even at concentrations as low as 5 μM (data not shown). We thus employed the novel pan-HSP90 inhibitor, the non-quinone PU-H71, already successfully used in cancer cell cultures and *in vivo* to reduce up-regulation of HSP90 and to inhibit the expression of Akt in a dose-dependent manner [26, 27]. PU-H71 was added to cell cultures 15 min. before the addition of Grp94-IgG at the final concentrations of both 50 nM (IC_{50}) and 150 nM, to measure to what extent effects of Grp94-IgG were dependent on the PI3K/Akt pathway activation. Stimulation of HSP90 expression by Grp94-IgG was inhibited by PU-H71 in a dose-dependent manner, as demonstrated by immunoblotting on whole cell lysates (Fig. 6A). A much higher, dose-dependent inhibitory effect was observed with PU-H71 on the HSP90 secretion in culture media where HSP90 was barely detectable already at 50 nM PU-H71 (Fig. 6B). It is worth noting that the same effect was also present with PU-H71 alone, as if the inhibition of HSP90 as such could lead to the block of HSP90 secretion.

PU-H71 at its lower concentration did not reduce significantly the Akt expression in cell lysates treated with Grp94-IgG (data not shown), whereas markedly inhibited Akt and P-Akt at 150 nM (Fig. 7). No substantial modification in the Akt activity was instead noted with PU-H71 alone. Results suggest that PU-H71 can antagonize the effects induced by Grp94-IgG on cellular HSP90, mostly affecting the delivery to cell membrane and secretion of HSP90 from the cell. This would imply interference with mechanisms of autocrine–paracrine activation of cell proliferation and angiogenic differentiation promoted by HSP90.

PU-H71 inhibits the MMP-9 pro-form expression and the angiogenic-like transformation of HUVECs induced by Grp94-IgG

The effective inhibition by PU-H71 of the HSP90 expression and secretion stimulated by Grp94-IgG would predict antagonism on the angiogenic-like promoting activity of Grp94-IgG. In addition, because MMP-9 is actively involved in the angiogenic process, and its stimulation by Grp94-IgG is mediated by HSP90-dependent Akt activation, it would be expected that the expression of MMP-9 were similarly inhibited by PU-H71. Experiments of zymography showed that PU-H71 at the highest concentration caused a significant reduction in the secretion of MMP-9 pro-form stimulated by Grp94-IgG (Fig. 8). This result indirectly proved that the Akt pathway was involved in the process of MMP-9 transport to the membrane and secretion from the cell that is functional to the diffusion of angiogenesis.

Optical microscopy inspection also revealed that PU-H71 was able to restore, in a concentration-dependent manner, the normal morphology of HUVECs, the tubule-like formations induced by Grp94-IgG being completely antagonized at 150 nM PU-H71 (Fig. 9).

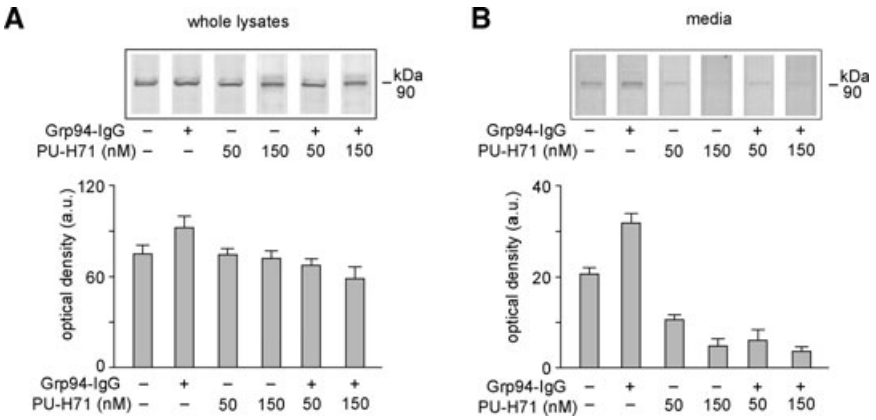

Fig. 6 PU-H71 inhibitor inhibits the HSP90 expression and secretion stimulated by Grp94-IgG complexes. Cells (25×10^4) were seeded in duplicate wells (2 ml) in absence (control) and presence of Grp94-IgG complexes (10 ng/ml) and PU-H71 at the indicated concentrations as specified in 'Materials and methods'. After 18 hrs incubation, supernatant was collected and cells lysed, as indicated in the legend to Figure 4, for obtaining whole lysates and measuring the expression of HSP90 in Western blotting (**A**). The same quantity of proteins was loaded in each lane without the addition of β-mercaptoethanol. (**B**) The media collected from cultured cells that were analysed for the expression of HSP90 (**A**), were also submitted to Western blotting for HSP90. Fifteen microlitres of media were loaded in each lane (without the addition of β-mercaptoethanol). In both (**A**) and (**B**), a representative blotting of three other independent analyses, is presented. Below each blotting is the graph with histograms representing the mean (±S.D.) of densitometric analysis of bands measured in all experiments ($n = 3$).

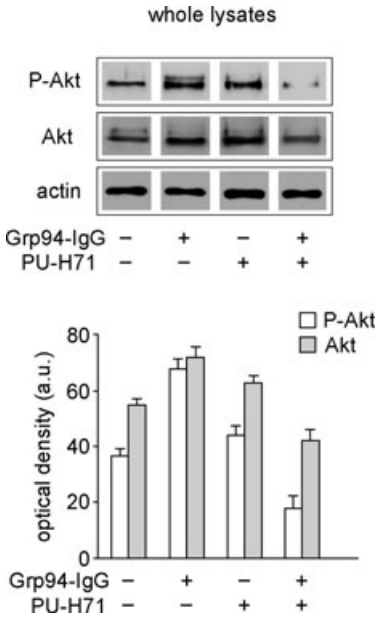

Fig. 7 PU-H71 inhibitor antagonizes the stimulation of Akt induced by Grp94-IgG complexes. Cells (25×10^4) were cultured as specified in 'Materials and methods' and after a 18 hrs incubation in absence (control) and presence of Grp94-IgG complexes (10 ng/ml) and PU-H71 (150 nM), both alone and together, were lysed and whole lysates treated with β-mercaptoethanol tested for the expression of total and P-Akt. A representative blotting of three other separate analyses is presented. Histograms in the graph below the blottings represent the mean (±S.D.) of densitometric analysis of bands measured in all experiments ($n = 3$).

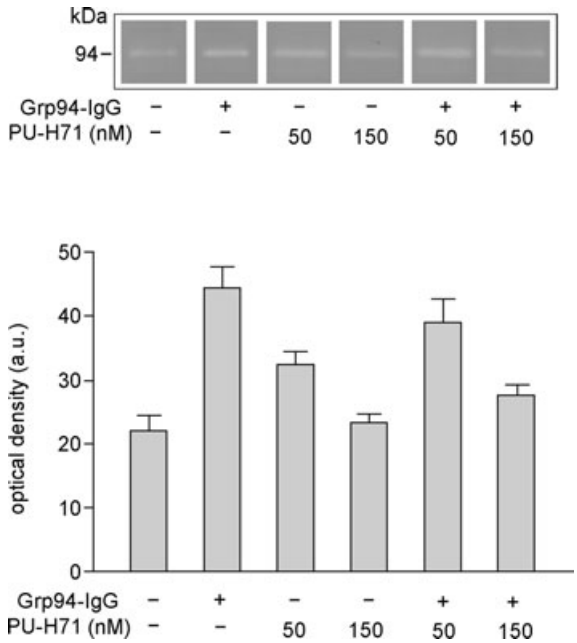

Fig. 8 PU-H71 inhibitor inhibits the secretion of MMP-9 stimulated by Grp94-IgG complexes. Media collected from cells (25×10^4) cultured in the presence of Grp94-IgG complexes (10 ng/ml) and PU-H71 (50 and 150 nM), both alone and together, were treated as specified in 'Materials and methods' and analysed in gel zymography for gelatinase activity. A representative gel zymography of three independent experiments is reported, showing the digestion band referred to the inactive, 94 kD MMP-9 pro-form, activated experimentally by zymography. Fifteen microlitres of media were loaded in each lane. Below is the graph with histograms representing the mean (±S.D.) of densitometric analysis of digestion bands measured in all experiments ($n = 3$).

Discussion

In this work we tested the selective MMP-2 and MMP-9 inhibitor, the cyclic decapeptide CTT as a potential inhibitor of proliferation and angiogenic-like transformation of HUVECs induced by complexes of Grp94-IgG. The choice of CTT was dictated by the previous observations showing that effects of complexes Grp94-IgG on

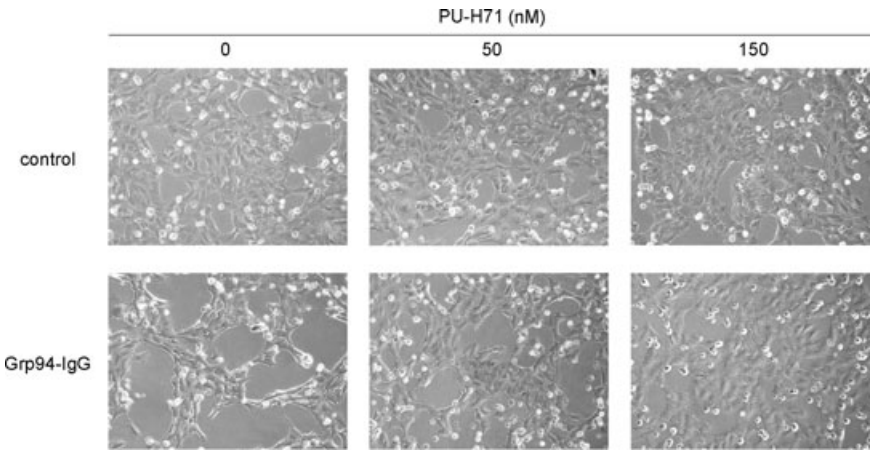

Fig. 9 PU-H71 inhibitor completely reverses the morphological alteration induced by Grp94-IgG complexes on HUVECs. Cells (25×10^4) were cultured in duplicate wells (2 ml) with Grp94-IgG (10ng/ml) and PU-H71 (50 and 150 nM), both alone and together, and after 18 hrs incubation morphology was visualized at the optical microscope. Representative pictures of each condition are presented with original magnification of 10×.

HUVECs were mediated by an increase in the expression and activity of MMP-9 [11], and MMPs are known to play a crucial role in sustaining angiogenesis and endothelial cell migration in pathological conditions [2, 6]. Grp94-IgG complexes isolated from plasma of diabetic subjects were identified as a marker of increased risk for developing vascular alterations that in time lead to stable and severe complications characterizing diabetes [10]. Thus, because CTT was proven effective in blocking angiogenesis and invasion of the blood vessel wall in tumour cells, thanks to specific anti-gelatinase activity [6, 13], morphological alterations induced by Grp94-IgG on endothelial cells might also be expected to be inhibited by CTT, thus supporting the use of CTT as anti-angiogenic agent also in conditions characterized by an increased concentration of these circulating complexes [10].

Results allowed us to address in depth the still unmet question of the molecular pathway specifically targeted by Grp94-IgG complexes for inducing morphological alterations in endothelial cells [11]. At the same time, we faced unexpected paradoxical biological effects of CTT. In particular, not only was CTT unable to inhibit angiogenic-like effects on HUVECs, but it even displayed a dose dependent, pronounced capacity to induce angiogenic transformation of cells (Fig. 1). This effect, apparently negating any anti-angiogenic activity, might be explained by considering that CTT is able to bind to structures on the cell membrane that activate mechanisms of intracellular signal transduction, similar to those involved by Grp94-IgG complexes. Although CTT homing to the vasculature in tumours has been explained by its binding to MMP-9 over-expressed on endothelial cells of newly formed vessels [13], hydrophobic nature of CTT, including its crucial HWGF sequence [13], can *per se* justify binding at sites in the membrane other than the specific membrane-bound target of MMP-9, as also supported by experiments demonstrating incorporation of CTT into phospholipids in liposomes [15]. Although to our knowledge no other MMP inhibitor has been observed to have a clear-cut angiogenic activity on HUVECs as CTT did, it is worth mentioning that a paradoxical cell growth stimulating and pro-angiogenic property is reported to occur also with tissue inhibitors of metalloproteases and other synthetic MMP inhibitors on certain cancer cell lines [28]. Importantly also, these unexpected, unwanted effects appeared to be independent of the MMP-inhibitory activity [1, 28], being instead mediated by specific structures on the cell membrane [28]. Inspection of the molecular mechanisms underlying the apparent synergic effect of CTT on angiogenic transformation by Grp94-IgG, excluded that the MEK-ERK1/2 pathway played a major role. This conclusion was supported by the following observations. First, CTT *per se* did not affect this pathway, although it turned out to enhance the intense ERK1/2 phosphorylation promoted by Grp94-IgG (Fig. 2), a result that confirmed the capacity of Grp94-IgG to directly stimulate the MEK/ERK1/2 pathway [11]. In addition, the specific MEK-ERK1/2 inhibitor U0126, while reducing the degree of ERK1/2 phosphorylation, did not change at all the morphological alterations induced by Grp94-IgG and CTT, both alone and together (data not shown). It was thus apparent that the differentiation effect of Grp94-IgG on HUVECs was mediated by another signalling transduction pathway also targeted by CTT, and that CTT was able to enhance the ERK1/2 phosphorylation driven by Grp94-IgG by activating this pathway (Fig. 2).

We present experimental evidence showing that the PI3K/Akt pathway is targeted by both Grp94-IgG and CTT, and that angiogenic-like effects on HUVECs are promoted by an HSP90-dependent activation of this pathway. Both Grp94-IgG and CTT were able to increase cellular expression of Akt and P-Akt, although Grp94-IgG was much more effective than CTT in this respect (Fig. 2). Intriguingly, however, when added together, the stimulation due to Grp94-IgG was reduced, although the expression of Akt remained still higher than the control. This result did not fit the picture of morphological alterations that even worsened when CTT was added to Grp94-IgG (Fig. 1). Because Akt pathway plays a pivotal role in mediating angiogenic effects [19, 25], a reduction of Akt expression would be expected to lead to a parallel reduction in morphological alteration. To search for a mechanism accounting for this contradictory result, we focused the attention on HSP90, because HSP90 is actively involved in mediating effects of Grp94-IgG on HUVECs [11]. HSP90 is the

chaperone of a lot of intracellular proteins [29–31], among which are those recruited in the crucial process of cell proliferation and differentiation [30–33], including also MMPs [1]. Akt is a client protein of HSP90 and a functional HSP90 is required for transduction of growth factor signalling through the Akt pathway [21]. Therefore, the inhibitory effect of CTT on Akt expression stimulated by Grp94-IgG might also be explained by reduction in either the expression or availability of HSP90. Experiments of immunofluorescence and immunoblotting on cell lysates-both whole and native lysates-clearly demonstrated that cell expression of HSP90 was slightly increased, not reduced, under the stimuli of Grp94-IgG and CTT, both alone and together (Fig. 4). However, whereas in the presence of both Grp94-IgG and CTT alone, HSP90 was almost entirely confined to cell membrane in podosomes (Fig. 3), the association led to a marked decrease in membrane-bound HSP90 (Figs 3 and 4). Thus, both Grp94-IgG and CTT might promote angiogenic transformation of HUVECs by stimulating the expression of HSP90 that was confined to the membrane in podosomes, where it is present in complexes with structural proteins-that account for intense structural modifications of actin cytoskeleton during differentiation of endothelial cells [31, 32]-and with proteins destined to secretion, such as MMPs [1, 10]. Several reports document that both active and inactive forms of MMP-9 are present in podosomes following the application of mitogenic stimuli [8, 34–36], and shedding of MMP-9 is responsible for proteolytic activity functional to paracrine diffusion of morphogenic alterations during angiogenesis [8, 34].

Although we were unable to measure MMP-9 expression in cell lysates-MMP-9 is hardly detected as protein in HUVECs [12]-the experiments of gel zimography clearly demonstrated that MMP-9 is intensely secreted in its inactive form in the presence of Grp94-IgG and CTT, and that association further increased the MMP-9 secretion (Fig. 5). This finding, together with the observation that also HSP90 underwent similar intense secretion, mostly when CTT was added to Grp94-IgG (Fig. 4), support the proposal that CTT enhances the angiogenic effect of Grp94-IgG by promoting the secretion of complexes of HSP90 with MMP-9, both concurring in reinforcing and propagating the mechanisms of angiogenic transformation and cell migration. Thus, reduction of both cellular Akt and membrane-bound HSP90 observed when CTT is added to Grp94-IgG does not mean that effects of Grp94-IgG are antagonized and that CTT might be taken as an effective inhibitor of Grp94-IgG on HUVECs. Results of immunofluorescence experiments rather indicate that CTT in the presence of Grp94-IgG alters the intra-cellular availability of HSP90, by accelerating its secretion. Although we did not investigate in details this specific molecular aspect, it is tempting to speculate that an increased demand of HSP90 for chaperoning cellular MMP-9 that is stimulated by both Grp94-IgG and CTT (Fig. 5) might partially and temporarily divert HSP90 from forming complexes with Akt that might thus be destabilized [21].

We offered a further proof that the angiogenic-like effect of Grp94-IgG is entirely sustained by an HSP90-dependent activation of the PI3K/Akt pathway, by using an HSP90 inhibitor, the non-quinone PU-H71 that down-regulates the PI3K/Akt pathway. PU-H71 does not affect cell viability, at variance with the classic PI3 kinase inhibitor LY294002 that in our cell culture drastically reduced cell viability even at the lowest concentration of 5 μM (data not shown). By occupying the ATP-binding site in HSP90, PU-H71 induces structural conformation in HSP90 that prevents binding of client proteins and their proper folding and activation [37, 38]. This mechanism is exploited for blocking overexpression of oncoproteins in cancer cells that require HSP90 for establishing their malignant phenotype, and purine-scaffold inhibitors of HSP90 are emerging as promising anti-tumour agents in clinical practice [39]. We observed that PU-H71 reduced in a concentration-dependent manner the stimulation of cellular HSP90 and Akt by Grp94-IgG, and in addition caused a marked fall in the secretion of HSP90 (Figs 6 and 7). This last effect was also displayed by PU-H71 alone, a result that suggested a mechanism by which the structural modification that HSP90 undergoes following inhibition prevents it from reaching the membrane and being secreted. This mechanism should also be supported by the observation that, in parallel with inhibition of HSP90 secretion, PU-H71 caused a fall in the MMP-9 secretion (Fig. 8), thus confirming that HSP90 is the chaperone of MMP-9 and that complexes of HSP90 with MMP-9 are delivered to the membrane and secreted during angiogenesis. In accord with the proposal that secretion of HSP90 and MMP-9 should be relevant for the induction of angiogenesis, and that blocking this secretion should also inhibit angiogenesis, we demonstrated that PU-H71 was able to reverse in a concentration-dependent manner the angiogenic transformation induced by Grp94-IgG, restoring completely the normal morphology in HUVECs (Fig. 9).

One would argue that effects observed with PU-H71 might be partly (if not entirely) due to inhibition of the ATP binding site on Grp94 rather than (or in addition to that) on HSP90, considering that PU-H71 is a pan-HSP90 inhibitor. However, different observations rule out this possibility. First, the availability of ATP binding site on Grp94 is strongly limited, if not precluded by steric constraint of Grp94 in the complex [11]. Indeed, at variance with what occurs with free Grp94, the addition of ATP to the complex does not modify the Grp94 conformation at all (personal observation). Second, PU-H71 is added to cell cultures 15 min. before the addition of Grp94-IgG, a procedure that permits the inhibitor to enter the cells and bind to HSP90, as demonstrated by the inhibition of HSP90 secretion by PU-H71 in absence of Grp94-IgG (Fig. 8). Finally, both the steric hindrance of the complex and the fact that its effects on HUVECs are mediated by a cell-surface receptor activation [11] favour the extracellular localization of Grp94-IgG rather than its internalization, being thus Grp94 also spatially prevented from taking contact with the inhibitor.

In conclusion, our work has revealed the essential role of HSP90 in the activation of the PI3K/Akt pathway that is targeted by Grp94-IgG for displaying the angiogenic-like effect on HUVECs. HSP90 appears as a promising therapeutic target for antagonizing the Akt-mediated mechanisms of activation of angiogenesis,

because it also supports the activation and secretion of MMP-9. Incidentally, we discovered paradoxical effects of the MMP-9 inhibitor CTT that on its own exhibited angiogenic-like property. This raises doubts about the claimed role of CTT as inhibitor of angiogenesis in pathological conditions [40]. Although the mechanisms underlying these effects of CTT on HUVECs deserve further investigation, our present results may nevertheless offer a molecular basis for explaining the disappointing effects observed with MMP inhibitors [2, 5, 28, 41] and that drastically hampered the use of MMP inhibitors, including CTT, as effective anti-angiogenic and anti-tumour agents [41].

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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