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Effect of Heparin Affin Regulatory Peptide on the Expression of Vascular Endothelial Growth Factor Receptors in Endothelial Cells

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Abstract. *Background: Heparin affin regulatory peptide (HARP) is an 18-kDa secreted protein that has been implicated in tumor growth and angiogenesis, although the mechanisms involved remain largely unknown. In the present work, the effect of human recombinant HARP on the expression of the vascular endothelial growth factor (VEGF) receptors KDR, Flt-1 and neuropilin-1 was studied in cultured human umbilical vein endothelial cells (HUVEC). Materials and Methods: The mRNA and protein levels of VEGF receptors were estimated by semi-quantitative RT-PCR and Western blot, respectively. Cell proliferation and migration were measured by MTT, direct counting of the cells and modified Boyden chamber assays. Results: HARP decreased the expression of KDR but increased the expression of Flt-1 and neuropilin-1 at both the mRNA and protein level. The effect reached a maximum 4 h after the addition of HARP into the cell culture medium and was reversed at later time-points. When HARP was added to the culture medium 4 h before the addition of $VEGF_{165}$, it inhibited $VEGF_{165}$-induced proliferation and migration of HUVEC. Conclusion: These data suggest that HARP affects the expression of VEGF receptors and inhibits $VEGF_{165}$-induced activation of HUVEC.*

Heparin affin regulatory peptide (HARP), also called pleiotrophin or heparin binding-growth associated molecule, is an 18-kDa secreted protein with high affinity for heparin and belongs to a family of heparin-binding growth factors, structurally distinct from the fibroblast growth factor family. Its amino acid sequence is highly conserved among species and contains two distinct lysine-rich clusters within both the NH_2- and COOH-terminal domains (1).

HARP was initially purified from bovine uterus (2) and neonatal rat brain (3) and displays important functions in the growth and differentiation in developing tissues, as well as during adulthood. HARP exhibits neurite outgrowth action in neonatal rat brain cells and mitogenic activity in some types of cells. HARP may also be an important regulator of tumor transformation, since it is detected in various carcinomas, is constitutively expressed in cell lines derived from these tumors and is involved in tumor growth, metastasis and angiogenesis (1, 4, 5). The mechanism of action of HARP has not yet been clarified and it is not clear whether its angiogenic action is due to a direct effect on endothelial cell processes, such as proliferation, migration and differentiation to form tubes (4, 6-8), or due to an effect on peripheral blood mononuclear cells (9), which in turn may activate the angiogenic process.

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic growth factors and plays a significant role in both development and homeostasis. In addition to its role in the activation of endothelial cells during the initial steps of angiogenesis, VEGF is also very important for the maintenance of the differentiated state of blood vessels (10). Many promoters or inhibitors of angiogenesis modulate VEGF or/and its receptors (11-13) and efforts to design drugs capable of interfering with VEGF signaling and blocking of tumor angiogenesis are intense (14).

Most of the VEGF activities are thought to be mediated by its interaction with two high affinity receptor tyrosine kinases, vascular endothelial growth factor receptor-2 (KDR in humans) and vascular endothelial growth factor receptor-1 (Flt-1) (15). Both Flt-1 and KDR are expressed on vascular endothelium and are essential for normal vascular

Abbreviations: HARP, heparin affin regulatory peptide; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor.

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development (16, 17). A third VEGF receptor, neuropilin-1, is also expressed by endothelial cells *in vivo* and *in vitro* and appears to be a mediator of angiogenesis (18).

In the present work, the effect of HARP on the expression of VEGF receptors was studied in cultured human umbilical vein endothelial cells (HUVEC) and on the $VEGF_{165}$-induced proliferation and migration of HUVEC.

Materials and Methods

Cell culture. HUVEC were isolated from human umbilical cords and cultured as previously described (6). The cells were grown as monolayers in M199 culture medium supplemented with 15% fetal calf serum (FCS), 200 μg/ml endothelial cell growth supplement, 4 U/ml heparin sodium, 100 U/ml penicillin-streptomycin and 50 μg/ml gentamycin and were used at passages 1-3. Cultures were maintained at 37˚C, 5% CO_2 and 100% humidity.

Purification of human recombinant growth factors. Expression of human recombinant HARP was induced in *E. coli* BL21 pLys cells transformed with the human HARP-pETHH8 plasmid, as previously described (6, 7). Human recombinant $VEGF_{165}$ was expressed in Sf9 insect cells and purified by cation exchange and heparin-affinity chromatography, as previously reported (19).

RNA isolation. Total RNA isolation was performed by the acid guanidinium isothiocyanate-phenol-chloroform method. After a 16-h incubation in M199 containing 5% FCS, the cells were treated with HARP (100 ng/ml) in the same medium for various time periods. They were then washed with ice-cold PBS and homogenized in 4 M guanidinium, 25 mM sodium citrate, 0.5% sarcosyl and 0.2 M beta-mercaptoethanol, by passing them several times through an 18-gauge syringe needle. Each homogenate was kept at –80˚C until used. Total RNA was extracted and its integrity was tested by running an aliquot on a 2.2 M formaldehyde, 1% agarose gel, as previously described (13).

Reverse transcription-polymerase chain reaction (RT-PCR). Primers used for human KDR, Flt-1 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were previously described (13). Primers for neuropilin-1 were designed according to the human sequence available (Accession Number AF018956).

cDNA primer	sense	anti-sense primer	product size
KDR	CCATTCCAC CAAAAGATG	AGACTTTGA GCATGGAAG	312 bp
Flt-1	GATGTCGACGGT ATAAATACCATG TGCTTCTAG	CTATGGAAGA TCTGATTTCT TACAGT	1081 bp
neuropilin-1	ATTCTGACC AGATCACAGC	CACCATACCC AACATTCC	455 bp
GAPDH	TCTAGACGGCAG GTCAGGTCCACC	CCACCCATGGCA AATTCCATGGCA	598 bp

The RT-PCR reactions for all the above mRNAs were performed in a single step, with 200-250 ng of total RNA, using the Access RT-PCR system (Promega) according to the following conditions: The reverse transcriptase reaction was performed by AMV-RT for 1 h at 48˚C. After an initial denaturation step for 2 min at 94˚C, 20-40 cycles of amplification (94˚C for 1 min, 55˚C for 1 min and 68˚C for 1 min) were performed and ended with a final DNA synthesis step at 68˚C for 10 min. In all cases, PCR reactions were not in the saturating phase (data not shown). DNA contamination was excluded by performing PCR reactions in the absence of the reverse transcription step.

The RT-PCR products of all the reactions were subjected to electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide and photographed using a digital camera. The ratio of electrophoretic band values of each PCR product *versus* GAPDH electrophoretic band values represents the relative expression of each gene at different time points after HARP application.

Western blot. After a 16-h incubation in M199 containing 5% FCS, HUVEC were treated with HARP (100 ng/ml) in the same medium for various time periods. They were then directly lysed in Laemmli sample buffer (0.03 M SDS, 0.05 M Tris base, 10% glycerol, 20% bromophenol blue and 10% mercaptoethanol) and the lysates were centrifuged at 13,000 g for 4 min. Eighty μl of each sample were loaded on 5% (for KDR and Flt-1) or 10% (for neuropilin-1 and actin) SDS-PAGE mini gels, analyzed and transferred to Immobilon P membranes (Millipore). Blocking was performed for 2 h at room temperature under continuous agitation by incubating the PVDF membranes with: a) 3% (w/v) bovine serum albumin (BSA, Sigma, Greece) in Tris-buffered saline pH 7,4 containing 0,05% Tween-20 (TBS-T) for KDR, b) Superblocker (Pierce, USA) for Flt-1, c) 5% (w/v) non-fat dry milk in TBS-T for neuropilin-1 and d) 3% (w/v) non-fat dry milk in TBS-T for actin. The membranes were then incubated overnight at 4˚C under continuous agitation with a monoclonal anti-human KDR antibody (Santa Cruz Biotechnology, Inc.) at a dilution 1:500 in 1% BSA in TBS-T, or a polyclonal anti-Flt-1 antibody (Santa Cruz Biotechnology) at a dilution 1:200 in 10% Superblocker in TBS-T, or a polyclonal anti-neuropilin-1 antibody (Santa Cruz Biotechnology) at a dilution of 1:500 in 3% non-fat dry milk in TBS-T or a monoclonal anti-human actin antibody (Chemicon, USA) at a dilution 1:500 in 3% non-fat dry milk in TBS-T. The mambranes then incubated for 1 h at room temperature under continuous agitation with peroxidase conjugated goat anti-mouse IgG (Sigma) at a dilution 1:5,000 in 1% BSA in TBS-T for KDR, or goat anti-rabbit IgG (Sigma) at a dilution 1:2,500 in 10% Superblocker in TBS-T for Flt-1, or goat anti-rabbit IgG at a dilution 1:10,000 in 3% non-fat dry milk in TBS-T for neuropilin-1 or goat anti-mouse IgG at a dilution 1:5,000 in 3% non-fat dry milk in TBS-T for actin. Detection of immunoreactive bands was performed by the ChemiLucent Detection System Kit (Chemicon), according to the manufacturer's instructions. The protein levels that corresponded to the immunoreactive bands were quantified using the ImagePC image analysis software (Scion Corporation, Frederick, MD, USA). The ratio of electrophoretic band values of each protein *versus* actin electrophoretic band values represents the relative amounts of each receptor at different time points after HARP application.

Cell proliferation assays. In order to determine if HARP affects VEGF-induced proliferation of HUVEC, the 3-[4, 5-dimethylthiazol-

2-yl]-2, 5-dimethyltetrazolium bromide (MTT) assay was used, as previously described (6). Briefly, cells were seeded at a density of $2x10^4$ cells/well in 24-well tissue culture plates and grown in complete medium. After 24 h, the medium was replaced with M199 containing 5% FCS. After an overnight incubation, the medium was replaced with fresh M199 containing 5% FCS, as well as the tested agents and the cells were further incubated for 48 h. When combinations of VEGF and HARP were used, HARP was added 4 h prior to VEGF. At the end of the incubation period with the tested agents, MTT stock (5 mg/ml in PBS) at a volume equal to 1/10 of the medium was added and plates were incubated at 37˚C for 2 h. The medium was removed, the cells were washed with PBS pH 7.4 and 100 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) were added to each well and agitated thoroughly, in order to solubilize the dark blue formazan crystals. The solution was transferred to a 96-well plate and immediately read on a microplate reader, at a wavelength of 490 nm. Results were always confirmed by direct measurement of the cells using a standard hemocytometer.

Migration assays. Migration assays were performed as previously described (7), in 24-well microchemotaxis chambers (Costar), using untreated polycarbonate membranes with 8 μm pores. After an overnight incubation of cultured HUVEC with M199 containing 5% FCS, cells were pre-incubated or not for 4 h with HARP (100 ng/ml) in M199 containing 5% FCS. They were then harvested and resuspended at a density of 105 cells/0.1 ml, in medium containing 0.25% BSA. The bottom chamber was filled with 0.6 ml of medium containing 0.25% BSA and HARP (100 ng/ml), VEGF (5 ng/ml) or their combination. The upper chamber was loaded with 10^5 cells and incubated for 4 h at 37˚C. After completion of the incubation, the filters were fixed with saline-buffered formalin and stained using DiffQuick. The cells that migrated through the filter were quantified by counting the whole area of each filter, using a grid and an Optech microscope at a 20X magnification.

Statistical analysis. The significance of variability between the results of each group and its corresponding control was determined by unpaired *t*-test or ANOVA. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean±S.E.M. from at least three independent experiments.

Results

Effect of HARP on the mRNA levels of VEGF receptors in HUVEC. The effect of human recombinant HARP on the mRNA amounts of VEGF receptors in HUVEC was investigated first. Subconfluent cells were cultured for 1, 2, 4 or 8 h in the presence or absence of HARP. Semi-quantitative RT-PCRs were performed using total RNA and specific primers for KDR, Flt-1, neuropilin-1 and GAPDH, as described in the Materials and Methods. HARP decreased KDR mRNA levels. The decrease was evident 2 h after addition of HARP in the cell culture medium, reached a maximum of about 20% after 4 h and was reversed at later time-points (Figure 1A and B). In contrast, HARP increased Flt-1 mRNA amounts 4 h after addition of the growth factor in the cell culture medium. The increase was about 20% and was reversed at later time-points (Figure 1A and C). HARP also increased neuropilin-1 mRNA amounts 2 h after it was added to the cell culture medium. The increase was also maximal (approximately 45%) at 4 h and was reversed at later time-points (Figure 1A and D).

Effect of HARP on protein levels of VEGF receptors in HUVEC. In order to see if HARP alters VEGF receptor protein levels, HUVEC were cultured for 2, 4 or 8 h in the presence or absence of HARP. Total crude protein extracts were analyzed using Western blotting techniques, as described in the Materials and Methods. As shown in Figure 2A, HUVEC express an immature form of KDR/Flk-1 with a molecular mass of about 150 kDa, a partially glycosylated intermediate with a molecular mass of about 200 kDa and a mature, fully glycosylated receptor with a molecular mass of about 230 kDa (20). HARP decreased the amounts of all three forms of KDR. The decrease was statistically significant for the fully glycosylated receptor in the first 4 h after the addition of HARP to the HUVEC medium and the maximal decrease (57%) was observed at 8 h (Figure 2A and B). The other two forms of KDR were decreased only 8 h after application of HARP. In contrast to the effect on KDR, HARP increased the amounts of Flt-1. The increase was evident 2 h after the addition of HARP in the HUVEC medium and the maximal increase (43%) was observed at 4 h, while this effect was reversed at later time-points (Figure 2A and C). Similarly, HARP increased the protein amounts of neuropilin-1. The increase was statistically significant in the first 2 h after addition of HARP to the HUVEC medium and the maximal increase (40%) was observed at 4 h, while the effect was reversed at later time-points (Figure 2A and D).

HARP suppressed $VEGF_{165}$-induced proliferation and migration of HUVEC. The effect of HARP on the $VEGF_{165}$-induced proliferation and migration of HUVEC was investigated. HUVEC were seeded in 24-well plates, as described in the Materials and Methods and HARP was added at concentrations of 30, 100 or 300 ng/ml. After 4 h of incubation, $VEGF_{165}$ was added at a concentration of 5 ng/ml and the cells were further incubated for 48 h. $VEGF_{165}$ significantly increased the number of HUVEC, while HARP partially inhibited $VEGF_{165}$-induced proliferation in a concentration-dependent manner. The maximum inhibition was approximately 40% when 300 ng/ml HARP were used (Figure 3).

The transfilter assay was used to study the effect of HARP on the $VEGF_{165}$-induced migration of HUVEC. As shown in Figure 4, $VEGF_{165}$ (5 ng/ml) significantly increased HUVEC migration. HARP inhibited HUVEC migration and completely reversed the stimulatory effect of $VEGF_{165}$. When cells were pre-incubated with HARP, the stimulatory effect of $VEGF_{165}$ was significantly impaired

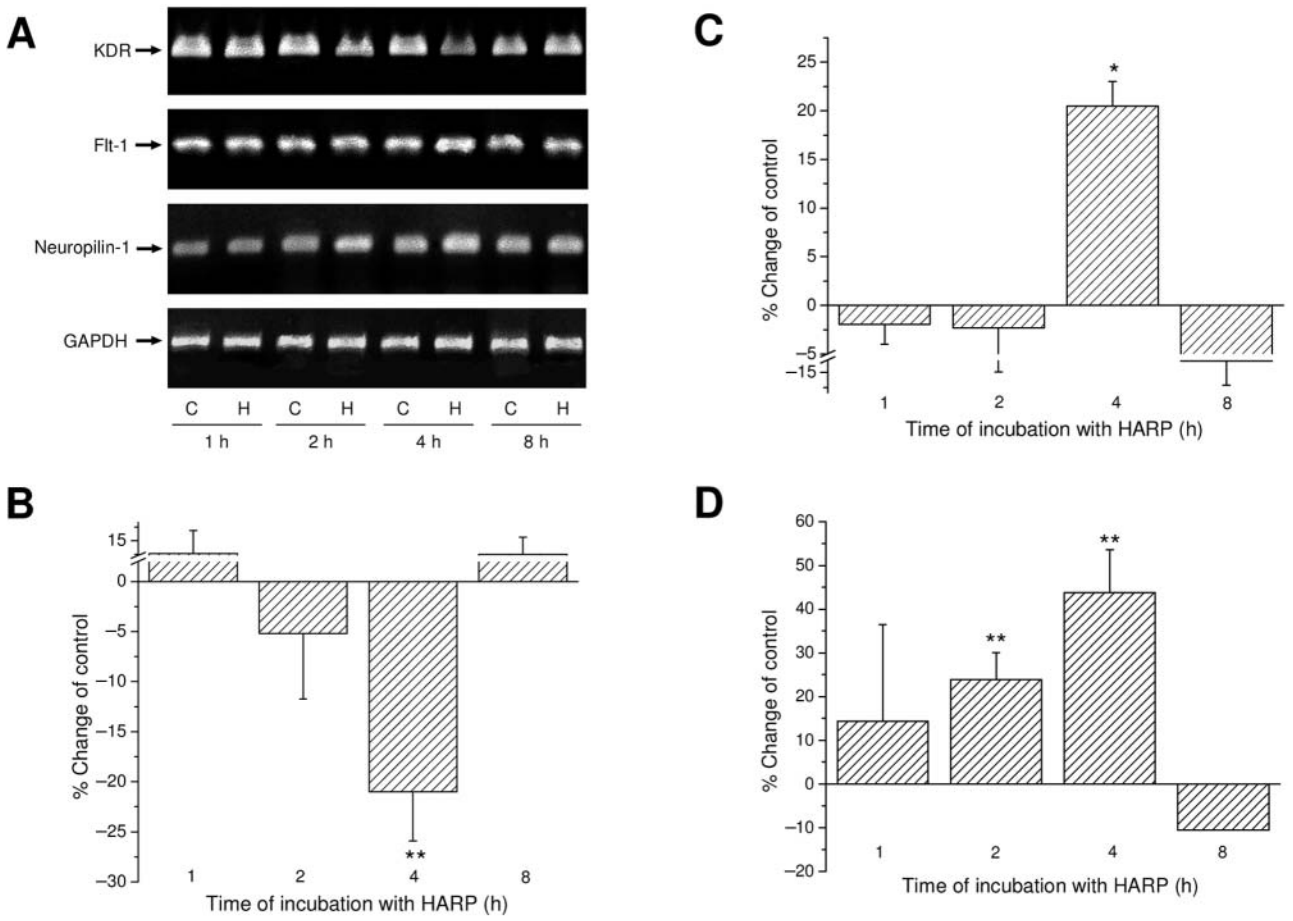

Figure 1. *HARP modulated the mRNA levels of VEGF receptors in HUVEC. (A) Products of RT-PCRs for the mRNAs of KDR, Flt-1, neuropilin-1 and GAPDH at several time-points after addition of HARP (100 ng/ml) to the HUVEC medium. C: Control; H: Cells treated with HARP. Representative picture of 4 independent experiments. The mRNA amounts were quantified by densitometric analysis of the corresponding bands and the ratio KDR/GAPDH (B) Flt-1/GAPDH (C) or neuropilin-1/GAPDH (D) mRNAs was calculated for each lane. Results are expressed as mean±S.E.M. of the % change of the KDR, Flt-1 or neuropilin-1 mRNA relative amounts in cells treated with HARP compared with the non-treated cells (control). Asterisks denote a statistically significant difference from the control (*p<0.05, **p<0.01).*

and was further decreased when HARP was also added in the lower chamber of the transfilter.

Discussion

Although considerable data have accumulated on the contribution of HARP on angiogenesis (1, 4, 6-8), the mechanisms by which it affects vessel growth in several systems have not yet been clarified. It is possible that HARP has a direct effect on endothelial cells (6-8), alters the effects of other angiogenic growth factors (21) or acts on other cell types which secondarily affect angiogenesis (9). The different forms of HARP that may be present in several systems or under certain circumstances (1, 5, 6, 22) may also differentially affect angiogenic processes (22). In the present work, we studied the effect of HARP on the expression of VEGF receptors and on the VEGF-induced proliferation and migration of HUVEC.

KDR is considered the main VEGF receptor involved in $VEGF_{165}$-induced endothelial cell proliferation and migration (15, 23, 24). Whether the negative effect of HARP on the expression of KDR reported here is direct or secondary, due to the direct binding of HARP to the $VEGF_{165}$ present in the serum (5% in our experiments), leading to decreased amounts of the latter, is not clear from the present study. Blocking $VEGF_{165}$ binding to KDR was shown to lead to decreased vascular KDR expression (25).

Although both Flt-1 and KDR are selectively expressed on vascular endothelium and are essential for normal vascular development (16, 17), Flt-1 is not considered to be involved in VEGF-induced endothelial cell proliferation

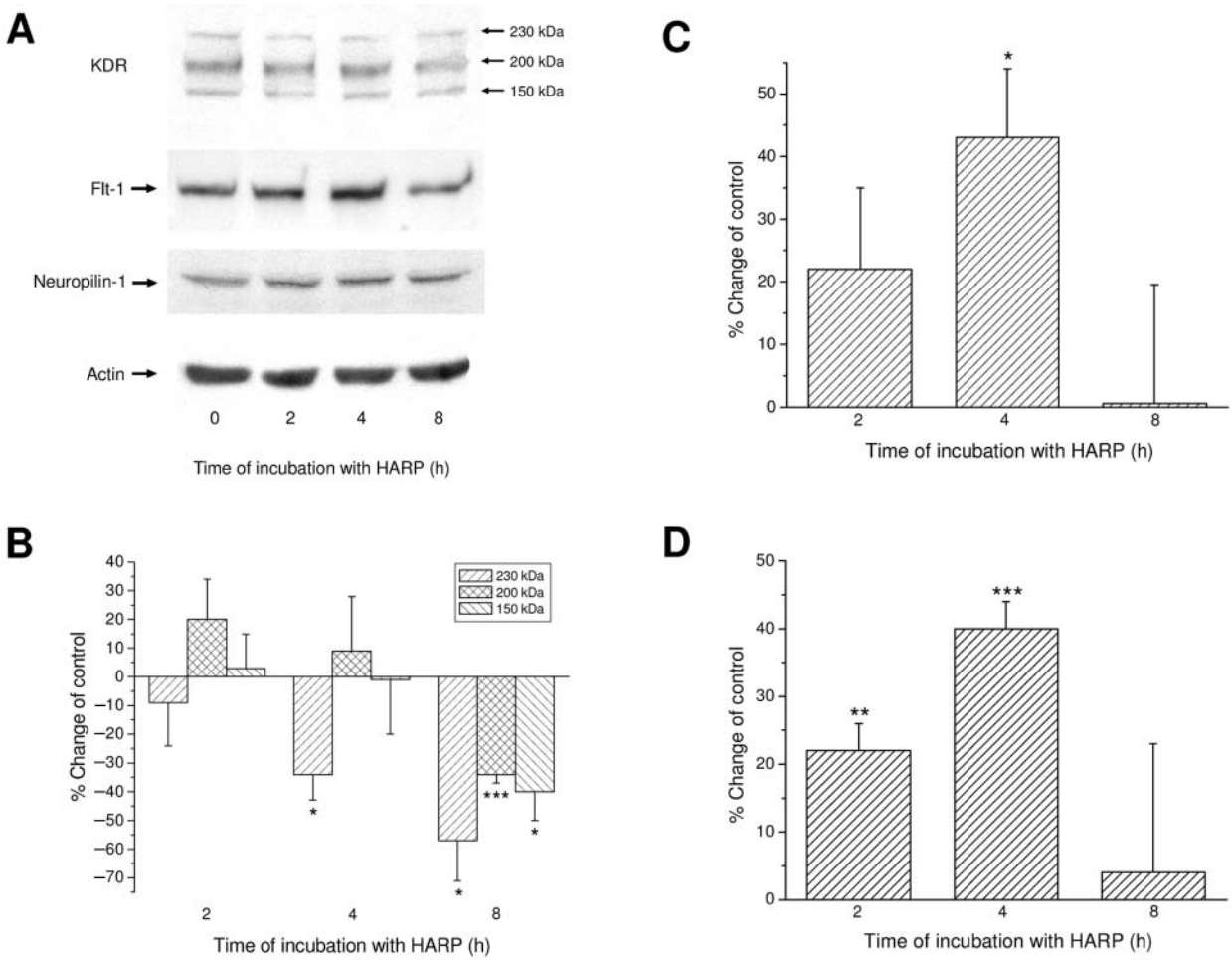

Figure 2. *HARP modulated the protein levels of VEGF receptors in HUVEC. (A) Western analysis for KDR, Flt-1, neuropilin-1 and actin at several time-points after addition of HARP (100 ng/ml) to the HUVEC medium. Representative picture of 3 independent experiments. The protein amounts were quantified by densitometric analysis of the corresponding bands and the ratio KDR/actin (B) Flt-1/actin (C) or neuropilin-1/actin (D) was calculated for each lane. Results are expressed as mean±S.E.M. of the % change of the KDR, Flt-1 or neuropilin-1 protein relative amounts in cells treated with HARP compared with the non-treated cells (control). Asterisks denote a statistically significant difference from the control (*p<0.05, **p<0.01, ***p<0.001).*

and migration (15, 23, 24). The prevalent hypothesis is that Flt-1 binding of VEGF leads to a decrease in the VEGF levels, which are available to interact with KDR and, thus, negatively regulate VEGF-induced angiogenesis (26, 27). It has also been recently suggested that Flt-1 modulates KDR signaling during blood vessel formation (28) and, thus, mice lacking Flt-1 died from vascular overgrowth, caused primarily by aberrant endothelial cell division (29). HARP down-regulated the expression of KDR and increased the expression of Flt-1, both of which may be mechanisms that lead to the inhibition of the $VEGF_{165}$-induced endothelial cell proliferation and migration.

In addition to the above mentioned mechanisms, Flt-1 also interacts with neuropilin-1 through its extracellular domain and regulates its levels available for binding to VEGF, again acting as a negative regulator of endothelial cell activation (30). Neuropilin-1 is a 130-135-kDa cell surface glycoprotein expressed by endothelial cells *in vivo* and *in vitro* and appears to be a mediator of angiogenesis (18). It is highly expressed by certain tumor cell types as the only VEGF receptor that contributes to tumor angiogenesis (31) and can independently promote cell signaling and migration, but not proliferation, in endothelial cells (32). Neuropilin-1 is also a co-receptor of KDR and enhances binding as well as the affinity of VEGF for KDR (18). VEGF selectively upregulates neuropilin-1 through KDR and neuropilin-1 is a therapeutic target for the suppression of pathological angiogenesis (33). In the present study, HARP down-regulated KDR expression and inhibited VEGF-induced

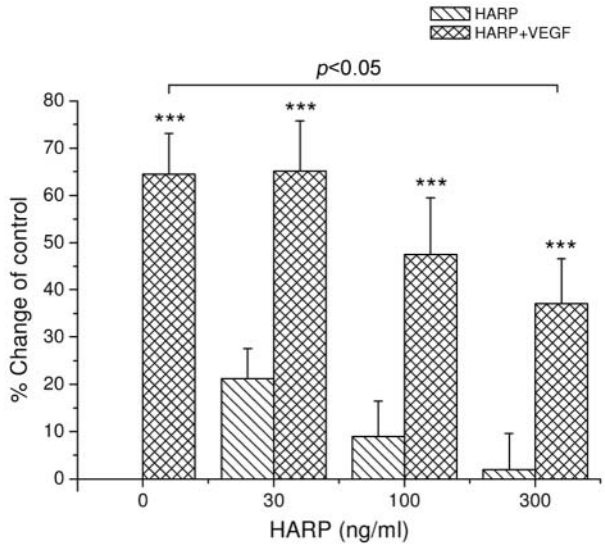

Figure 3. *Effect of HARP on VEGF-induced proliferation of HUVEC. Different amounts of HARP were added to the culture medium of HUVEC 4 h before addition of VEGF (5 ng/ml). Forty-eight hours later, the number of cells was estimated as described in the Materials and Methods. Results are expressed as mean±S.E.M. of the % change of the number of cells compared with the untreated cells (not stimulated with HARP or VEGF). Asterisks denote a statistically significant difference from the untreated cells (***p<0.001).*

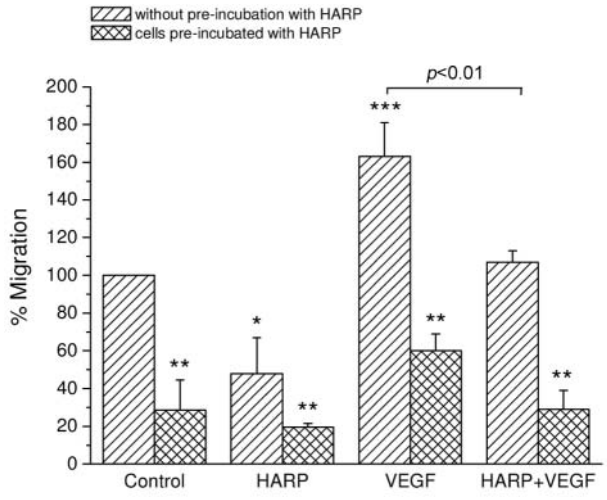

Figure 4. *Effect of HARP on VEGF-induced migration of HUVEC. Migration was measured using the transfilter assay, as described in the Materials and Methods. Results are expressed as mean±S.E.M. of the % change compared with the untreated cells (control without pre-incubation with HARP). Asterisks denote a statistically significant difference from the untreated cells (*p<0.05, **p<0.01 and ***p<0.001).*

proliferation and migration of HUVEC. Therefore, it is possible that HARP directly increases the expression of neuropilin-1, which, however, does not seem to adequately counteract the inhibitory effect of HARP on $VEGF_{165}$-induced HUVEC activation. The latter could partially be due to increased neuropilin-1 binding by the increased amounts of Flt-1, as discussed above.

We have previously shown that HARP negatively regulates the angiogenic activity of $VEGF_{165}$ by forming a complex with it (21). In the present work, we showed that HARP inhibited $VEGF_{165}$-induced proliferation and migration of HUVEC perhaps due to a modulation of the expression of VEGF receptors. Whether the effect of HARP on each VEGF receptor is direct or indirect is not clear from the present study and both cases could be possible.

To date, the only functional HARP receptor identified on HUVEC is the receptor protein tyrosine phosphatase β/ζ (8), which mediates HARP-induced migration in the absence of serum. In the presence of serum, however, HARP leads to a small decrease of HUVEC migration (present study and 7), possibly due to inhibition of the effect of VEGF or other growth factors present in the serum. It seems, therefore, that the effect of HARP on endothelial cell functions largely depends on the microenvironment of the cells.

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