Biochemical and Biophysical Research Communications **282,** 306–313 (2001)
doi:10.1006/bbrc.2001.4574, available online at http://www.idealibrary.com on IDEAL®

HARP Induces Angiogenesis *in Vivo* and *in Vitro:* Implication of N or C Terminal Peptides

E. Papadimitriou,* A. Polykratis,† J. Courty,‡ P. Koolwijk,§ M. Heroult,‡ and P. Katsoris†,[1]

**Laboratory of Molecular Pharmacology, Department of Pharmacy, and †Division of Genetics, Cell and Developmental Biology, Department of Biology, University of Patras, Patras, Greece; ‡Laboratory of CRRET, University of Paris Val de Marne, Avenue du Général de Gaulle, 94010 Créteil, France; and §Gaubius Laboratory TNO-PG, Zernikedreef 9, 2333 CK Leiden, The Netherlands*

Received February 15, 2001

HARP (heparin affin regulatory peptide) is a growth factor displaying high affinity for heparin. In the present work, we studied the ability of human recombinant HARP as well as its two terminal peptides (HARP residues 1–21 and residues 121–139) to promote angiogenesis. HARP stimulates endothelial cell tube formation on matrigel, collagen and fibrin gels, stimulates endothelial cell migration and induces angiogenesis in the *in vivo* chicken embryo chorioallantoic membrane assay. The two HARP peptides seem to be involved in most of the angiogenic effects of HARP. They both stimulate *in vivo* angiogenesis and *in vitro* endothelial cell migration and tube formation on matrigel. We conclude that HARP has an angiogenic activity when applied exogenously in several *in vitro* and *in vivo* models of angiogenesis and its NH_2 and COOH termini seem to play an important role. © 2001 Academic Press

Key Words: **HARP; angiogenesis; endothelial cells; chicken embryo chorioallantoic membrane.**

HARP (heparin affin regulatory peptide), also called pleiotrophin or HB-GAM (heparin binding-growth associated molecule), is a 18 kDa secreted protein with distinct lysine-rich clusters within both the NH_2- and COOH-terminal domains (1). HARP has a high affinity for heparin and is localised in the extracellular matrix through interactions with glycosaminoglycans (2–5). It is highly conserved among species (2, 4, 6) and shares 50% homology with midkine and the avian retinoic-induced heparin binding protein (2, 7, 8). These proteins constitute a new heparin-binding growth factor family, structurally distinct from the FGF family (8).

[1] To whom correspondence should be addressed at Division of Genetics, Cell and Developmental Biology, Department of Biology, University of Patras, Patras, GR26504 Greece. Fax: 30-61-994797. E-mail: katsopan@upatras.gr.

HARP is a developmentally regulated protein that exhibits neurite outgrowth action in neonatal rat brain cells (1, 2, 9). Concerning its mitogenic activity, controversial results are reported, that vary according to the type and the origin of the studied cells (10–14). HARP was initially purified from bovine uterus (15) and neonatal rat brain (1). It is expressed in developing tissues (1, 16) and displays important functions in the growth and differentiation processes. In adults, HARP has been found in neuronal tissues, heart, uterus, cartilage and bone (17, 18), indicating that it may as well have important physiological roles during adulthood. HARP may also be an important regulator of tumour transformation. It is detected in various carcinomas (human breast and prostate cancer, neuroblastomas, benign meningiomas, small cell lung cancer, rat mammary tumours), exhibiting a protooncogene function (13, 19). It is constitutively expressed in cell lines derived from these tumours and is involved in tumour growth and metastasis (19–21). Cells transformed by the HARP cDNA, when implanted into nude mice, form highly vascularised tumours. Moreover, many of the HARP expressing tumours become more invasive when HARP gene is overexpressed (17) and reduction of HARP levels reduces the number of blood vessels in primary tumours, indicating a possible action of HARP related to the blood vessel network formation of solid tumour mass (21).

We have recently demonstrated a mitogenic effect of human recombinant HARP expressed in bacterial cells when presented to endothelial cells as a substrate and this effect was mimicked by two peptides corresponding to its NH_2 and COOH termini (22). In the present study, we investigated the effect of the above mentioned HARP and peptides on angiogenesis, using a variety of *in vitro* and *in vivo* assays.

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVEC), rat adrenal medulla microvascular endothelial (RAME) cells, bovine

0006-291X/01 $35.00

retinal endothelial cells (BREC), bovine brain capillary (BBC) endothelial cells and human foreskin microvascular endothelial cells (hMVEC) were isolated and cultured as previously described (10, 22, 23). Cultures were maintained at 37°C, 5% CO_2 and 100% humidity.

Purification of human recombinant HARP and HARP peptides. Expression of the recombinant HARP was induced in *E. coli* BL21 pLys cells transformed with the human HARP-pETHH8 plasmid (kindly provided by P. Bohlen), as previously described (22). The bacterial product, similarly to the mammalian HARP, forms dimmers and oligomers that are stable in the presence of β-mercaptoethanol or dithiothreitol (data not shown). The formation of these covalent complexes is induced by transglutaminase, similarly to what has been described for midkine (24).

The sequences of the HARP peptides corresponding to the NH_2 and COOH terminus of the protein were NH_2-AEAGKKEKPEKKVKKSDCGEW-COOH (HARP residues 1–21) and NH_2-AESKKKKKEGKKQEKMLD-COOH (HARP residues 121–139) respectively, as previously described (22) and were obtained from (SYNT:EM, France).

Matrigel tube formation assay. The tube formation assay was performed as previously described (25). Briefly, matrigel was used to coat the wells of 24-well plates (0.25 ml/well) and was left to polymerise for 1 h at 37°C. After polymerisation, 40,000 cells suspended in 1 ml of the corresponding medium supplemented with FCS (2% for HUVEC and BBC cells and 10% for RAME cells, that wouldn't form any tubes at lower serum concentration) were added to each well. HARP or the peptides were added to the corresponding wells just prior to addition of the cells. After 18 h of incubation (except for RAME cells that were incubated only for 6 h because of tube regression at longer incubations), the medium was removed, the cells were fixed and the length of the tube network was measured as previously described (25).

Collagen tube forming assay. Three dimensional type I collagen gels were prepared as previously described, by mixing 8 vol of a solution of rat collagen type I (4 mg/ml) with 1 vol of DMEM 10× and 1 vol of sodium bicarbonate (2.2 g/l) at 4°C to prevent immediate gelation. 0.5 ml of the cold mixture was added to each well of a 24-well plate and allowed to gelify for 10 min at 37°C (26). 5×10^4 BREC or 2×10^4 BBC cells were seeded on each collagen gel of a 24-well plate in DMEM supplemented with 2% (BREC) or 10% (BBC cells) FCS and 1 ng/ml FGF-2. One day after reaching confluency, different concentrations of HARP or each one of the peptides were added to the cell culture medium. The reorganisation of the endothelial cell monolayer was monitored and photographed with an Olympus IMT-2 inverted phase contrast microscope equipped with a digital camera.

Fibrin tube forming assay. Human fibrin matrices were prepared as described (23). Highly confluent endothelial cells were detached and seeded in a 1.25:1 split ratio on the fibrin matrices and cultured for 24 h in M199 medium supplemented with 10% human serum, 10% NBCS, and penicillin/streptomycin. After 24 h, the medium was replaced with medium containing 100 ng/ml HARP, 10 ng/ml FGF-2, 50 ng/ml vascular endothelial growth factor A (VEGF-A), 5 ng/ml tumour necrosis factor (TNF), or combinations of these mediators and cultured for a period of 7–9 days. The culture medium with the mediators was refreshed every two or three days. Invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analysed by phase contrast microscopy. The total length of tube-like structures of six randomly chosen microscopic fields/well (7.3 mm^2/field = 44% of the well) was measured using an Olympus CK2 microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software.

Boyden chamber assay. Migration assays were performed as previously described (27), in a 24-well microchemotaxis chamber (Costar), using untreated polycarbonate membranes with 8 μm pores. HUVEC, BBC and RAME cells were harvested and resuspended at a concentration of 10^5 cells/0.1 ml, in the corresponding medium containing 0.25% bovine serum albumin (BSA). The bottom chamber was filled with 0.6 ml of the corresponding medium containing 0.25% BSA and HARP or each of the peptides. The upper chamber was loaded with 10^5 cells and incubated for 4 h at 37°C. FGF-2 was used as a positive control at a concentration of 5 ng/ml. 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 quantitated by counting the whole area of each filter using a grid and an Optech microscope at a 20× magnification.

The chicken embryo chorioallantoic membrane (CAM) assay. The *in vivo* CAM angiogenesis model (28) was used with minor modifications. Leghorn fertilised eggs (Pindos, Greece) were incubated for 4 days at 37°C, when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator until day 9, when the test molecules were applied. HARP or the peptides were added as a solution containing different amounts of the agents in a final volume of 20 μl of PBS, at day 9 of chicken embryo development on an area of 1 cm^2 of the CAM, restricted by a plastic ring. In a different group of eggs, which was used as control, only PBS was applied. After 48 h of incubation at 37°C, the CAMs were fixed *in situ* with saline-buffered formalin, excised from the eggs, placed on slides and left to air-dry. Pictures at a 2.5× magnification were then taken through a stereoscope equipped with a digital camera and the total length of the vessels was measured as described above for the matrigel tube formation assay. Assays for each test sample were carried out three times and each experiment contained 10–20 eggs per data point.

Statistical analysis. The significance of variability between the results from various groups was determined by one-way analysis of variance (ANOVA). Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean ± SEM from at least three independent experiments.

RESULTS

The ability of human recombinant HARP produced in bacterial cells and both peptides corresponding to its terminal domains to stimulate tube formation *in vitro* was tested using three different assays of angiogenesis, namely the collagen gel (26), the matrigel (25) and the fibrin gel (23).

HARP stimulates tube formation on collagen gels. When seeded on collagen gels, BBC endothelial cells formed a monolayer (Fig. 1, top, A). In the presence of HARP, there was a complete change of phenotype and the cells formed cord-like structures within 24 h, which were observed on and at several levels under the monolayer (Fig. 1, top, B). The formation of cord-like structures seemed to be concentration-dependent, starting at 10 ng/ml of HARP and reaching a plateau between 100 and 300 ng/ml. The effect was more intense when HARP was added in the mass of the collagen gel instead of being added in the cell culture medium. When either the NH_2 or the COOH peptide was added to the cell culture medium, there was only a weak stimulation, observed after several days (data not shown).

Human recombinant HARP added to the cell culture medium of BREC stimulated cord-like structures formation in a concentration-dependent way, with the

maximum effect observed between 100 and 300 ng/ml (Fig. 1, top, C and D, and bottom).

RAME cells or HUVEC plated on collagen gels did not survive and HARP did not improve their survival.

HARP stimulates tube formation on matrigel. The ability of HARP or either of the two peptides to promote tube formation was also tested on matrigel, which measures both migration and differentiation of the cells (25). BBC endothelial cells on matrigel did not form an extensive tubular network when cultured in low serum medium (Fig. 3A). HARP stimulated tube formation by BBC endothelial cells in a concentration-dependent manner, with a maximum effect observed at 300 ng/ml (Figs. 2A and 3A and 3B). Both HARP peptides stimulated tube formation by these cells in a way similar to the intact molecule (data not shown). On the contrary, HARP or each peptide had a small inhibitory effect on HUVEC tube formation, which was not statistically significant (Figs. 2B and 3C and 3D). In RAME cells, HARP or the peptides stimulated tube formation in a concentration-dependent manner, with maximal effect at 100 ng/ml (Figs. 2C and 3E and 3F).

HARP stimulates tube formation on fibrin gels. To investigate the ability of HARP or its terminal peptides to induce endothelial cell tube formation on fibrin gels, hMVEC were cultured on top of three-dimensional fibrin matrices. In this *in vitro* model, the combination of a growth factor (e.g. FGF-2 or VEGF-A) with the inflammatory mediator TNFα induces tube formation by the hMVEC (23), whereas these mediators by themselves are not effective (data not shown, ref. (23). HARP, either in the absence or presence of TNFα did not induce tube formation. However, HARP was found to have a marginally significant potentiating effect on FGF-2/TNFα or VEGF/TNFα induced tube formation in a concentration-dependent manner and the maximum effect was obtained with 100 ng/ml of HARP. There was an increase of 1.7 $\pm$ 0.29 folds (four independent experiments, $P < 0.05$) and 1.5 $\pm$ 0.35 folds (three independent experiments) in VEGF/TNFα- and FGF-2/TNFα-induced tube formation respectively, by the addition of HARP. Neither of the two terminal peptides of HARP had any effect on tube formation in this model, either alone or in combination with the other compounds (data not shown).

HARP stimulates RAME cell migration. The Boyden chamber test was used to estimate the migratory effect of HARP and the two peptides. As a positive control, FGF-2 was used at a concentration of 5 ng/ml (data not shown). All three molecules significantly induced migration of BBC (Fig. 4A) and RAME cells (Fig. 4B). HUVEC migration was not significantly affected by HARP or any of its peptides (Fig. 4C).

HARP induces angiogenesis in vivo. The chicken embryo CAM was used as a suitable *in vivo* model to

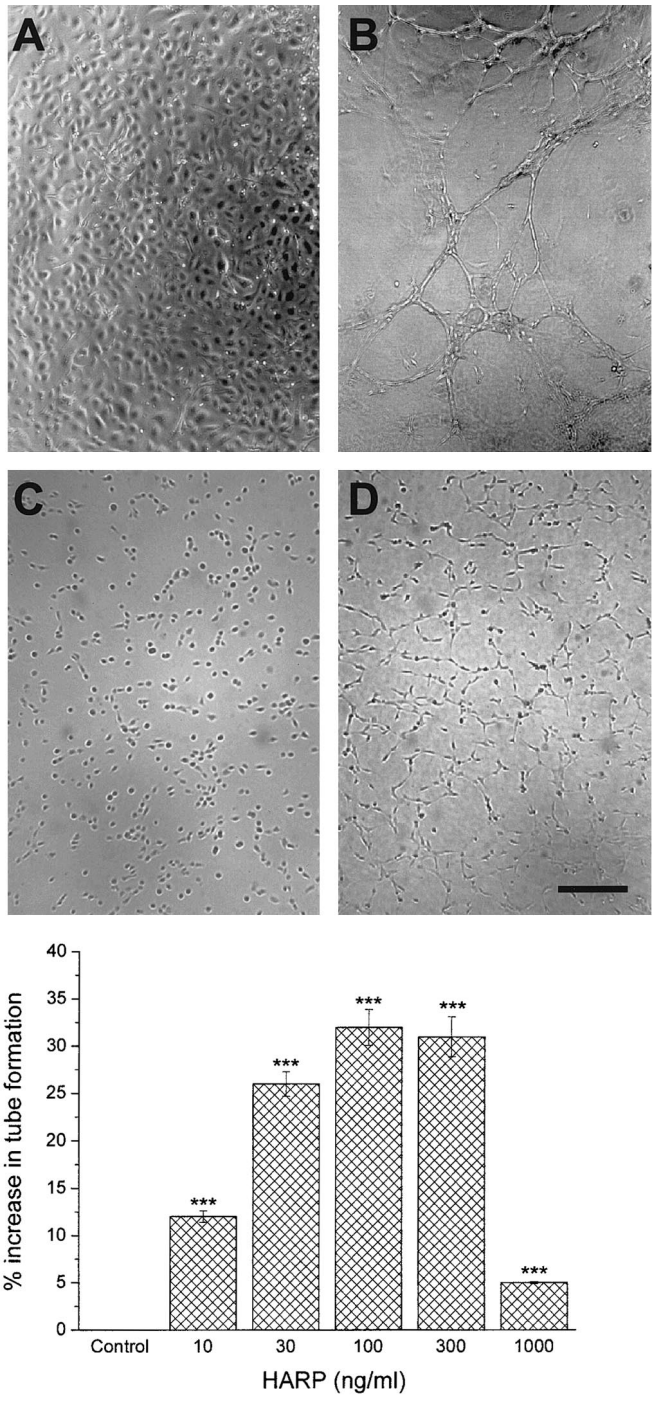


FIG. 1. (Top) Effect of human recombinant HARP on endothelial cells grown on collagen gels for 24 h (phase contrast microscopy). (A) Control BBC cells, (B) BBC cells treated with human recombinant HARP (100 ng/ml), (C) Control BREC, and (D) BREC treated with human recombinant HARP (100 ng/ml). Bar represents 500 μm. (Bottom) Effect of human recombinant HARP on BREC grown on collagen gels. Tube formation was assayed in the presence of varying amounts of HARP, as described under Materials and Methods. Total tube length was expressed as percent of the values obtained without stimulation. Data are the mean $\pm$ SEM of five independent experiments. ***$P < 0.001$.

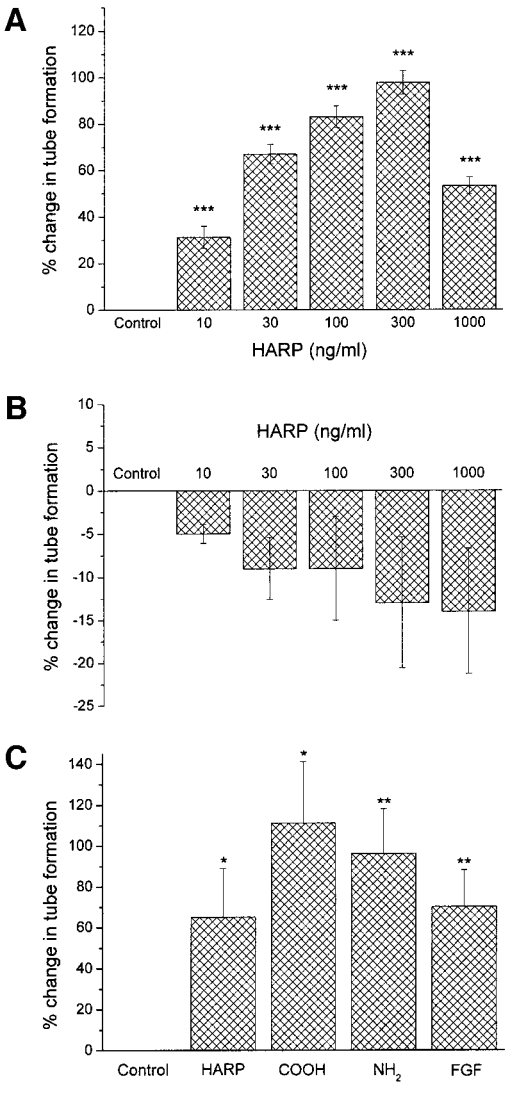


FIG. 2. Effect of human recombinant HARP on BBC cells (A) and HUVEC (B) grown on matrigel. Tube formation was assayed in the presence of varying amounts of HARP, as described under Materials and Methods. (C) Effect of human recombinant HARP and two peptides corresponding to its COOH- and NH_2-termini (100 ng/ml), on RAME cells grown on matrigel. Total tube length was expressed as percent of the values obtained without stimulation. Data are the mean ± SEM of three independent experiments. $***P < 0.001$.

study the angiogenic action of HARP and its terminal peptides. Different concentrations of molecules were tested and 48 h after effector molecule application, the total length of the vessel network was measured, as described under Materials and Methods. As can be seen in Fig. 5, HARP and the COOH peptide induced angiogenesis in a dose-dependent and statistically significant manner. The NH_2 peptide also had a weak angiogenic effect which is statistically significant at a dose of 0.5 μg/ring. These results indicate that HARP is a potent angiogenic factor *in vivo* and its NH_2 and COOH termini may participate in this biological action.

DISCUSSION

In the present study, we investigated the effect of human recombinant HARP expressed in bacterial cells and two synthetic peptides (HARP residues 1–21 and 121–139) on angiogenesis, using *in vitro* and *in vivo* experimental models. HARP has been shown to be

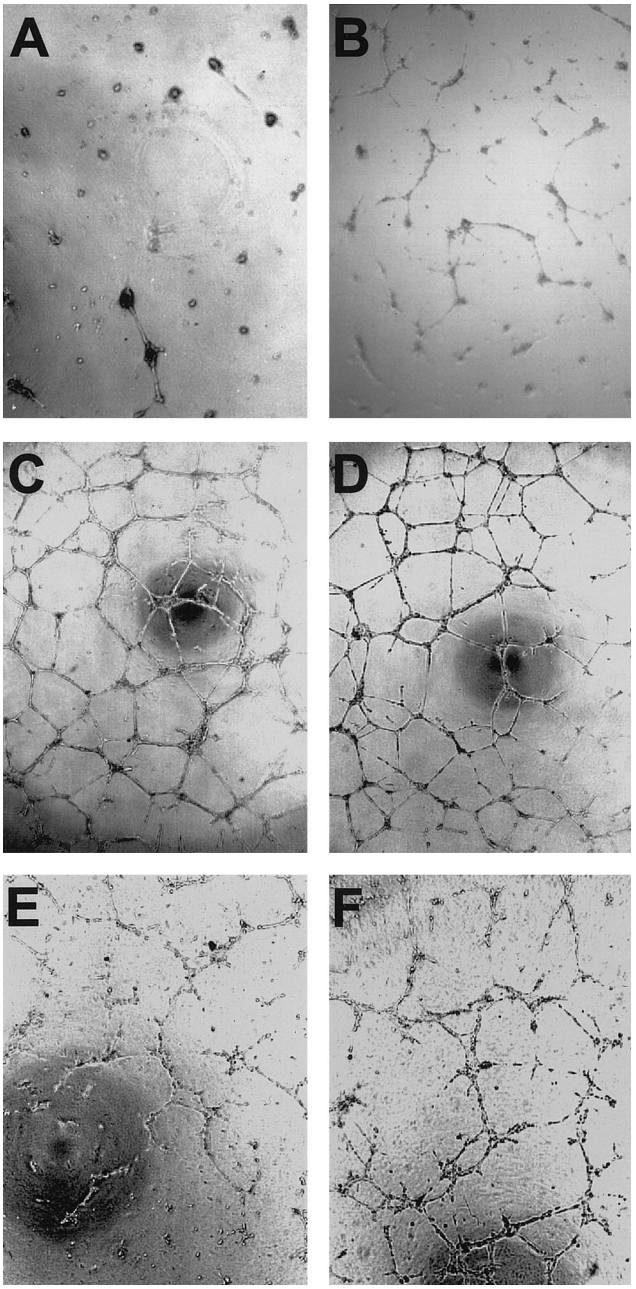


FIG. 3. Effect of human recombinant HARP on tube formation on matrigel (phase contrast microscopy). (A) Control BBC cells, (B) BBC cells treated with human recombinant HARP (100 ng/ml), (C) control HUVEC, and (D) HUVEC treated with human recombinant HARP (100 ng/ml). (E) Control RAME cells, (F) RAME cells treated with human recombinant HARP (100 ng/ml).

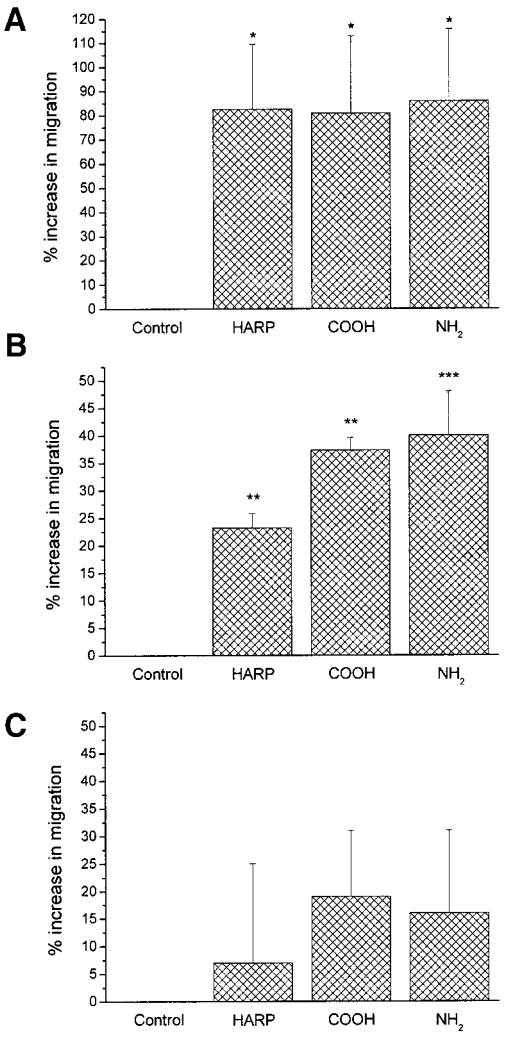
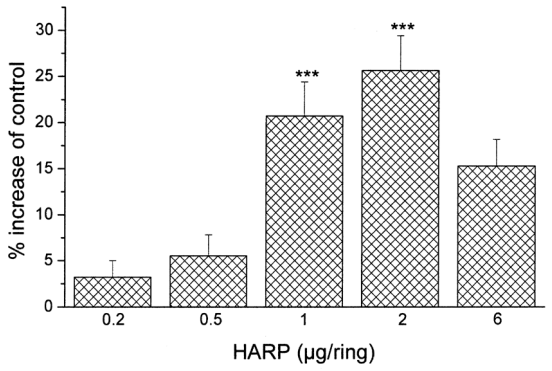


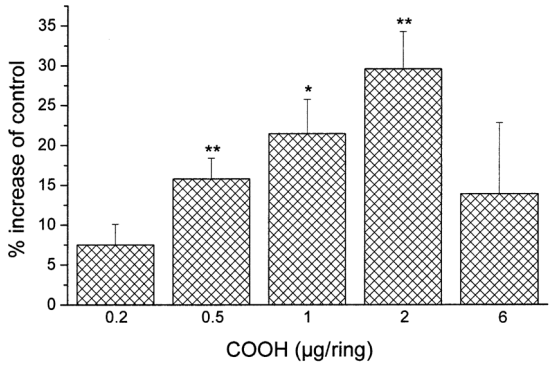
FIG. 4. Human recombinant HARP (100 ng/ml) and two peptides, corresponding to its COOH- and NH_2-termini (100 ng/ml), stimulate migration of BBC (A) and RAME cells (B) but not HUVEC (C). Results are expressed as percent of the values obtained without stimulation. FGF-2, which was used as a positive control, caused a 140% stimulation of migration (data not shown). Data are the mean ± SEM of at least three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

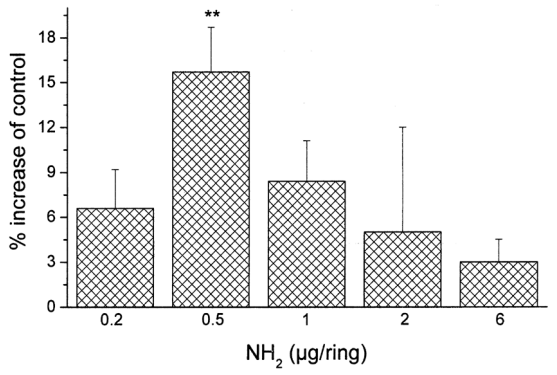
expressed in a variety of primary human tumours (13) and has been correlated with enhanced tumour growth and vascular density (21, 29). The notion that HARP was angiogenic has been previously relied on an increased vascularity of tumors that expressed HARP (21, 29), the ability of human recombinant HARP expressed in a eucaryotic system to induce bovine aortic endothelial cell tube formation on collagen gels (26), and the angiogenic activity of HARP transfectant MCF-7 cells in the rabbit corneal assay (29). However, there was no direct and extensive evidence that HARP itself is an angiogenic molecule. Angiogenesis implicates many different actions, such as cell adhesion, migration, proliferation, differentiation and degradation of the basal lamina. In order to demonstrate the possible angiogenic role of HARP, we tested the implication of HARP as well as its two terminal peptides in endothelial cell processes involved in angiogenesis.

We have recently demonstrated that human recombinant HARP expressed in bacterial cells acts as a mitogen for different endothelial cells, namely BBC, RAME and HUVEC, when presented to them as a substrate (22). The response of these cells to both HARP and its terminal peptides is different, with HUVEC being the less responsive (22 and present study). Different cell surface molecules may interact with HARP among the different cells, depending on their origin and function. HARP could not be detected either in the culture medium or in the extracellular matrix of HUVEC (22). Other large vessel endothelial cells, like those deriving from bovine aorta, secrete HARP in both the culture medium and the extracellular matrix (data not shown) and are responsive to stimulation by HARP (26).

HARP binds with high affinity to heparin and to glycosaminoglycans, including heparan sulphate (4, 5) and dermatan sulphate (5) derived from extracellular matrix, functioning possibly as a mitogenic molecule entrapped in the extracellular space. In the same line, it had a greater tube formation activity when incorporated into the collagen gels than when added in the medium of the cells, while when matrigel was used as an *in vitro* angiogenesis assay, the effect was the same whether HARP was in the medium or in the mass of matrigel (data not shown). The difference could be due to the different nature of the *in vitro* assays used to test the implication of HARP on endothelial cell tube formation. On collagen gels, the cells can attach, spread, proliferate and finally form tubes (26), while on matrigel they do not proliferate, but migrate and finally differentiate forming tubes (25). Moreover, matrigel contains by itself several growth factors and other constituents of the extracellular matrix, while collagen or fibrin gels do not. It seems that HARP stimulates tube formation in all of these systems, however through different mechanisms. This is further strengthened by the fact that the two peptides (HARP residues 1–21 and 121–139) have a stimulating effect on matrigel but no effect on collagen or fibrin gel assays. The two lysine-rich terminal domains of HARP are implicated in multiple biological actions, since they stimulate endothelial cell proliferation (22) and are required for successful transformation of NIH 3T3 cells by HARP residues 41–64 (30). In addition, we have recently showed that the C-terminal part of HARP (HARP residues 111–136) is clearly involved in both mitogenic and cellular transformation effect (31). Both terminal regions account for the ability of HARP to bind tightly to heparin and to extracellular matrix (2, 4), although it was recently suggested that binding to heparin occurs primarily to the β-sheet domains of HARP (32). It







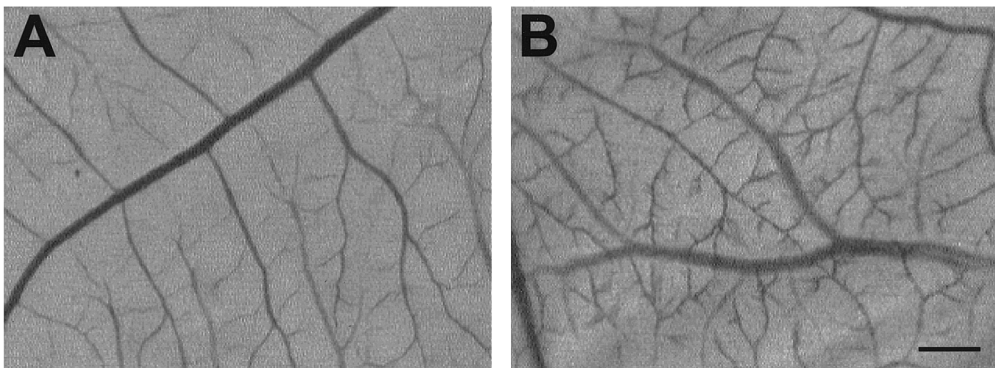


FIG. 5. Induction of angiogenesis by human recombinant HARP and two peptides, corresponding to its COOH- and NH_2-termini, in the chicken embryo CAM assay. (Top) Different quantities of tested molecules in the same final volume (20 μl) were applied on an area of 1 cm^2, restricted by a plastic ring, at CAMs of day 9, as described under Materials and Methods. After 48 h of incubation at 37°C, the CAMs were fixed, excised from the eggs, and photographed and the total length of capillaries network was measured. Data are the mean ± SEM of three independent experiments, each one with 20 eggs tested for each concentration used. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (Bottom) Representative pictures showing the CAM vascular network when unstimulated or stimulated with 2 μg of HARP. Bar represents 500 μm.

has been hypothesised that the positively charged NH_2 and COOH termini of HARP interact with the receptor or other cell surface molecules and facilitate HARP binding to a high affinity receptor (30).

In the present study, both HARP peptides had by themselves a significant effect, similar to the effect of HARP and in some cases seem to be active even at lower amounts than the whole molecule. As we have recently reported, HARP can form covalently bound dimmers and oligomers (33). Although the HARP domain involved in the dimerization process is unknown, the positively charged NH_2 and COOH termini of HARP, involved in its biological activity (22, 30), might be masked and unable to bind to the HARP receptor. Alternatively, in equal amounts of HARP and peptides, the concentration of the peptides used is higher than that of the whole molecule of HARP. Whether these or similar peptides exist physiologically (e.g. after proteolysis of HARP), is not known at present and is under investigation.

The mechanism of action of HARP is not yet clarified and it is not clear whether its angiogenic effect is due to interactions with cell surface proteoglycans or binding to another cell surface receptor. Several HARP receptors have been reported, the first being N-syndecan (syndecan-3), a transmembrane heparin sulphate proteoglycan which mediates the neurite-promoting signal (14). Another receptor for HARP is the receptor protein tyrosine phosphatase (RPTP)β/ζ (34, 35), which increases tyrosine phosphorylation through ligand-dependent receptor inactivation (35). Concerning HARP mitogenic activity, a high affinity binding site for HARP has been described in NIH 3T3 cells (36) and tyrosine phosphorylation seems to play an important role (37). No specific cell surface macromolecules related to HARP angiogenic activity have been yet identified. Glycosaminoglycans induce HARP dimerisation via covalent bonds, a key event in the activation of the transmembrane signalling receptors of several growth factors (33). It is possible that HARP interaction with endothelial cells may involve both glycosaminoglycans and a transmembrane specific receptor, although it is not clear if and to what extent these interactions are involved in the angiogenic effect induced by HARP. Moreover, it is possible that HARP or its terminal peptides have an indirect effect, potentiating the angiogenic effect of other growth factors or releasing growth factors sequestered in the ECM, such as FGF-2, by inducing stimulation of proteases (22, 38).

In conclusion, our findings emphasise the role of HARP in angiogenesis both *in vivo* and *in vitro*. HARP synthetic peptides representing native molecule residues 1–21 and 121–139 seem to be involved in the angiogenic activity of HARP, suggesting that minimal structures could be sufficient to trigger endothelial cell activation if they are suitably presented to the cell. Interestingly, the effect of HARP and both peptides was qualitatively different among the different angiogenic assays tested.

ACKNOWLEDGMENTS

This work was in part supported by grants from the Research Committee of the University of Patras (P.K., Karatheodoris), the Ministére de l'Education Nationale et de la Recherche (DRED/CNRS), the Association pour la Recherche sur le Cancer (J.C., N° 5257) and the Dutch Cancer Society (P.K., TNOP 95-1511). M.H. received a doctoral fellowship from "The association pour la Recherche sur la Polyarthrite." We are also grateful to the Private Maternity Clinic of Patras for providing us with umbilical cords.

REFERENCES

1. Rauvala, H. (1989) An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. *EMBO J.* **8,** 2933–2941.
2. Li, Y. S., Milner, P. G., Chauhan, A. K., Watson, M. A., Hoffman, R. M., Kodner, C. M., Milbrandt, J., and Deuel, T. F. (1990) Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. *Science* **250,** 1690–1694.
3. Ledoux, D., Caruelle, D., Sabourin, J. C., Liu, J. F., Crepin, M., Barritault, D., and Courty, J. (1997) Cellular distribution of the angiogenic factor heparin affin regulatory peptide (HARP) mRNA and protein in the human mammary gland. *J. Histochem. Cytochem.* **45,** 1239–1245.
4. Kinnunen, T., Raulo, E., Nolo, R., Maccarana, M., Lindahl, U., and Rauvala, H. (1996) Neurite outgrowth in brain neurons induced by heparin-binding growth-associated molecule (HB-GAM) depends on the specific interaction of HB-GAM with heparin sulfate at the cell surface. *J. Biol. Chem.* **271,** 2243–2248.
5. Vacherot, F., Delbe, J., Heroult, M., Barritault, D., Fernig, D. G., and Courty, J. (1999) Glycosaminoglycans differentially bind HARP and modulate its biological activity. *J. Biol. Chem.* **274,** 7741–7747.
6. Merenmies, J., and Rauvala, H. (1990) Molecular cloning of the 18-kDa growth associated protein of developing brain. *J. Biol. Chem.* **265,** 16721–16724.
7. Hampton, B. S., Marshak, D. R., and Burgess, W. H. (1992) Structural and functional characterization of full-length heparin-binding growth associated molecule. *Mol. Biol. Cell* **3,** 85–93.
8. Urios, P., Duprez, D., Le Caer, J.-P., Courtois, Y., Vigny, M., and Laurent, M. (1991) Molecular cloning of RI-HB, a heparin binding protein regulated by retinoic acid. *Biochem. Biophys. Res. Commun.* **175,** 617–624.
9. Raulo, E., Julkunen, I., Merenmies, J., Pihlaskari, R., and Rauvala, H. (1992) Secretion and biological activities of heparin-binding growth-associated molecule. *J. Biol. Chem.* **267,** 11406–11416.
10. Courty, J., Dauchel, M-C., Caruelle, D., Perderiset, M., and Barritault, D. (1991) Mitogenic properties of a new endothelial cell growth factor related to pleiotrophin. *Biochem. Biophys. Res. Commun.* **180,** 145–151.
11. Delbe, J., Vacherot, K., Laaroubi, D., Barritault, D., and Courty, J. (1995) Effect of heparin on bovine epithelial lens cell proliferation induced by heparin affin regulatory peptide. *J. Cell. Physiol.* **164,** 47–54.
12. Sato, H., Funahashi, M., Kristensen, D. B., Tateno, C., and Yoshizato, K. (1999) Pleiotrophin as a Swiss 3T3 cell-derived potent mitogen for adult rat hepatocytes. *Exp. Cell Res.* **246,** 152–164.

13. Zhang, N., and Deuel, T. F. (1999) Pleiotrophin and midkine, a family of mitogenic and angiogenic heparin-binding growth and differentiation factors. *Curr. Opin. Hematol.* **1,** 44–50.
14. Raulo, E., Chernousov, M. A., Carey, D. J., Nolo, R., and Rauvala, H. (1994) Secretion and biological activities of heparin-binding growth-associated molecule. Neurite outgrowth-promoting and mitogenic actions of the recombinant and tissue-derived protein. *J. Biol. Chem.* **269,** 12999–13004.
15. Milner, P. G., Li, Y.-S., Hoffman, R. M., Kodner, C. M., Siegel, N. R., and Deuel, T. F. (1989) A novel 17 Kd heparin-binding growth factor (BGF-8) in bovine uterus: purification and N-terminal amino acid sequence. *Biochem. Biophys. Res. Commun.* **165,** 1096–1103.
16. Bohlen, P., and Kovesdi, I. (1991) HBNF and MK members of a novel gene family of heparin-binding proteins with potential roles in embryogenesis and brain function. *Prog. Growth Factor Res.* **6,** 25–34.
17. Vanderwinden, J. M., Mailleux, P., Schiffmann, S. N., Vanderhaeghen, J. J. (1992) Cellular distribution of the new growth factor pleiotrophin (HB-GAM) mRNA in developing and adult rat tissues. *Anat. Embryol.* **186,** 387–406.
18. Mitsiadis, T. A., Salmivirta, M., Muramatsu, T., Muramatsu, H., Rauvala, H., Lehtonen, E., Jalkanen, M., and Thesleff, I. (1995) Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial–mesenchymal interactions during fetal development and organogenesis. *Development* **121,** 37–51.
19. Chauhan, A. K., Li, Y. S., and Deuel, T. F. (1993) Pleiotrophin transforms NIH 3T3 cells and induces tumors in nude mice. *Proc. Natl. Acad. Sci. USA* **90,** 679–682.
20. Zhang, N., Zhong, R., Wang, Z.-Y., and Deuel, T. F. (1997) Human breast cancer growth inhibited *in vivo* by a dominant negative pleiotrophin mutant. *J. Biol. Chem.* **272,** 16733–16736.
21. Czubayko, F., Schulte, A. M., Berchem, G. J., and Wellstein, A. (1996) Melanoma angiogenesis and metastasis modulated by ribozyme targeting of the secreted growth factor pleiotrophin. *Proc. Natl. Acad. Sci. USA* **93,** 14753–14758.
22. Papadimitriou, E., Heroult, M., Courty, J., Polykratis, A., Stergiou, C., and Katsoris, P. (2000) Endothelial cell proliferation induced by HARP: Implication of N or C terminal peptides. *Biochem. Biophys. Res. Commun.* **274,** 242–248, doi:10.1006/bbrc.2000.3126.
23. Koolwijk, P., Van Erck, M. G. M., De Vree, W. J. A., Vermeer, M. A., Weich, H. A., Hanemaaijer, R., and Van Hinsberg, V. W. M. (1996) Cooperative effect of TNFα, bFGF and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. *J. Cell Biol.* **132,** 1177–1188.
24. Kojima, S., Inui, T., Muramatsu, H., Suzuki, Y., Kadomatsu, K., Yoshizawa, M., Hirose, S., Kimura, T., Sakakibara, S., and Muramatsu, T. (1997) Dimerization of midkine by tissue transglutaminase and its functional implication. *J. Biol. Chem.* **272,** 9410–9416.
25. Pipili-Synetos, E., Papadimitriou, E., and Maragoudakis, M. E. (1998) Evidence that platelets promote tube formation by endothelial cells on matrigel. *Br. J. Pharmacol.* **125,** 1252–1257.
26. Laaroubi, K., Delbe, J., Vacherot, F., Desgranges, P., Tardieu, M., Jaye, M., Barritault, D., and Courty, J. (1994) Mitogenic and *in vitro* angiogenic activity of human recombinant heparin affin regulatory peptide. *Growth Factors* **10,** 89–98.
27. Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., Geppetti, P., and Ledda, F. (1994) Nitric oxide mediates angiogenesis *in vivo* and endothelial cell growth and migration *in vitro* promoted by substance P. *J. Clin. Invest.* **94,** 2036–2044.
28. Pipili-Synetos, E., Kritikou, S., Papadimitriou, E., Athanassiadou, A., Flordellis, C., and Maragoudakis, M. E. (2000) Nitric oxide synthase expression, enzyme activity and NO production during angiogenesis in the chick chorioallantoic membrane. *Br. J. Pharmacol.* **129,** 207–213.
29. Choudhuri, R., Zhang, H.-T., Donnini, S., Ziche, M., and Bicknell, R. (1997) An angiogenic role for neurokines midkine and pleiotrophin in tumorigenesis. *Cancer Res.* **57,** 1814–1819.
30. Zhang, N., Zhong, R., and Deuel, T. F. (1999) Domain structure of pleiotrophin required for transformation. *J. Biol. Chem.* **274,** 12959–12962.
31. Bernard-Pierrot, I., Delbe, J., Caruelle, D., Barritault, D., Courty, J., and Milhiet, P. E. (2001) The lysine-rich C-terminal tail of HARP is required for mitogenic and tumor formation activities. *J. Biol. Chem.*, in press.
32. Kilpelainen, L., Kaksonen, M., Kinnunen, T., Avikainen, H., Fath, M., Linhardt, R. J., Raulo, E., and Rauvala, H. (2000) Heparin-binding growth-associated molecule contains two heparin-binding β-sheet domains that are homologous to the thrombospondin type I repeat. *J. Biol. Chem.* **275,** 13564–12570.
33. Bernard-Pierrot, I., Heroult, M., Lemaitre, G., Barritault, D., Courty, J., and Milhiet, P. E. (1999) Glycosaminoglycans promote HARP/PTN dimerization. *Biochem. Biophys. Res. Commun.* **266,** 437–442, doi:10.1006/bbrc.1999.1835.
34. Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H., and Noda, M. (1996) 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase zeta/RPTPbeta, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *J. Biol. Chem.* **272,** 21446–21452.
35. Meng, K., Rodriguez-Pena, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., and Deuel, T. F. (2000) Pleiotrophin signals increased tyrosine phosphorylation of β-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase β/ζ. *Proc. Natl. Acad. Sci. USA* **97,** 2603–2608.
36. Kuo, M. D., Huang, S. S., and Huang, J. S. (1992) Characterization of heparin-binding growth-associated factor receptor on NIH 3T3 cells. *Biochem. Biophys. Res. Commun.* **182,** 188–194.
37. Souttou, B., Ahmad, S., Rigel, A. T., and Wellstein, A. (1997) Signal transduction pathways involved in the mitogenic activity of pleiotrophin. Implication of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways. *J. Biol. Chem.* **272,** 19588–19593.
38. Kojima, S., Inui, T., Kimura, T., Sakakibara, S., Muramatsu, H., Amanuma, H., Maruta, H., and Muramatsu, T. (1995) Synthetic peptides derived from midkine enhance plasminogen activator activity in bovine aortic endothelial cells. *Biochem. Biophys. Res. Commun.* **206,** 468–473.