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Endothelial Cell Proliferation Induced by HARP: Implication of N or C Terminal Peptides

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HARP (Heparin Affin Regulatory Peptide) is a 18-kDa secreted protein displaying high affinity for heparin. It has neurite outgrowth-promoting activity, while there are conflicting results regarding its mitogenic activity. In the present work, we studied the effect of human recombinant HARP expressed in bacterial cells as well as two peptides (HARP residues 1–21 and residues 121–139) on the proliferation of three endothelial cell types derived from human umbilical vein (HUVEC), rat adrenal medulla (RAME), and bovine brain capillaries (BBC) either added as a soluble form in the cell culture medium or coated onto the culture plate. HARP added in a soluble form in the culture medium had no effect on the proliferation of BBC, HUVEC, and RAME cells. However, when immobilized onto the cell culture plate, HARP had a concentration-dependent mitogenic effect on both BBC cells and HUVEC. The peptides presented as soluble factor induced a significant concentration-dependent mitogenic effect on BBC cells but only a small effect on HUVEC and RAME cells. When they were immobilized onto the cell culture plate, the mitogenic effect was much greater. The most responsive cells were BBC that expressed and secreted in the culture medium the higher amounts of HARP. © 2000 Academic Press

Key Words: **HARP; pleiotrophin; HB-GAM; endothelial cells.**

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 localized 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 (1, 2, 7). These proteins constitute a new heparin-binding growth factor family, structurally distinct from the FGF family (8). HARP is expressed in developing tissues (1, 2, 10, 12) and displays important function in the growth and differentiation processes. In adults, HARP has been found in neuronal tissues, heart, uterus, cartilage and bone (9–12), indicating that it may also have important physiological roles during adulthood. High levels of HARP are detected in speciments of many human tumors, including neuroblastoma, glioblastoma, prostate cancer, lung cancer and Wilms' tumor (13–18). *In vitro,* HARP mRNA is detected in various human cell lines, originally derived from breast cancer, prostate cancer and ovarian carcinoma (19–22).

HARP has an indisputable neurite outgrowth promoting activity (1, 2, 23) while there are conflicting results regarding its mitogenic activity (6, 24, 25), that vary according to the type and the origin of the studied cells (11, 14, 26–28). Only the recombinant polypeptide produced in a mammalian system displayed induction of cellular growth and has been shown to stimulate proliferation of fibroblasts, epithelial and endothelial cells (2, 11, 14). HARP expressed in insect cells or in a bacterial expression system has neurite outgrowth activity but in all studies up to date lacks mitogenic activity, suggesting that the mitogenic and the neurite outgrowth activities are mediated by distinct protein domains of the molecule (24, 29).

In the present study, we investigated the effect of human recombinant HARP expressed in bacterial cells and two peptides that correspond to the amino- and carboxyl-terminals of HARP on the proliferation of three endothelial cell types, when presented to the cells as a soluble factor or as a substrate. We also examined if these cells expressed and secreted HARP into their culture medium and/or deposited it onto their extracellular matrix.

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MATERIALS AND METHODS

Materials. Cell culture reagents were from Biochrom KG (Seromed, Germany). The affinity purified HARP antibodies were obtained as previously described (30). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins were obtained from Diagnostics-Pasteur (Marne la Coquette, France). Heparin-agarose was from Sigma, Immobilon P was from Millipore Corporation and the chemiluminescence developing system (ECL) was from Amersham (Pharmacia Biotech). The sequences of the HARP peptides corresponding to the NH_2 and the COOH terminus of the protein were NH_2-AEAGKKEKPEKKVKKSDCGEW-COOH (HARP residues 1–21) and NH_2-AESKKKKKEGKKQEKMLD-COOH (HARP residues 121–139) respectively and were of purity higher than 85% (SYNT:EM, France). All other reagents were of analytical grade and were purchased from Sigma.

Purification of human recombinant HARP. *E. coli* BL21 pLys cells transformed with the human HARP-pETHH8 plasmid (kindly provided by P. Bohlen), were cultured at 37°C in LB media containing 50 μg/ml ampicillin and 50 μg/ml chloramphenicol until a cell density with an absorbance 0.4 at 600 nm was achieved. Expression of the recombinant HARP was induced for 2 h at 37°C by the addition of 2 mM IPTG. Bacteria were then centrifuged and the pelleted cells were resuspended in 50 mM Tris HCl, pH 7.5 containing 1 mM EDTA, 1 μg/ml of leupeptin, 1 μg/ml pepstatin A and 1 μg/ml aprotinin. After three cycles of freezing/thawing, the lysate was sonicated with six pulses of 20 sec each, centrifuged at 27,000 g for 30 min at 4°C and the resulting pellet- representing insoluble material and inclusion bodies- was solubilized in 50 mM Tris, pH 8.0 containing 10 mM dithiothreitol, 0.1 M EDTA, 1 M NaCl and 8 M urea. After stirring overnight at 4°C, insoluble material was removed by centrifugation (27,000 g, 30 min) and the supernatant was dialyzed against 25 mM Hepes, pH 7.4 containing 1 M NaCl at 4°C for 8 h followed by a second 8 h dialysis against 25 mM Hepes, pH 7.4 at 4°C. After adjusting the ionic strength to 0.5 M NaCl, the refolded proteins were loaded on a heparin-Sepharose column at flow rate of 30 ml/h. After washing extensively with 50 mM Tris-HCl, pH 7.5 containing 0.5 M NaCl, the bound proteins were eluted with 50 mM Tris-HCl, pH 7.5 containing 2 M NaCl. The eluted proteins were then diluted with 50 mM Tris-HCl, pH 7.5 to an ionic strength of 0.4 M NaCl and loaded on a Mono S column (FPLC system, Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.5. Proteins were eluted from the Mono S column using a linear 0.4 to 1 M NaCl gradient at a flow rate of 0.5 ml/min in 50 mM Tris HCl, pH 7.5. Each collected fraction was quantified for its protein contents with a BCA assay (Pierce) using BSA as standard protein.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords, cultured as previously described (31) and used at passages 1–5. Rat adrenal medulla microvascular endothelial (RAME) cells were a kind gift of Dr. P. I. Lelkes (University of Wisconsin Medical School, WI), were cultured as previously described and used at passages 19–21 (32). Bovine brain capillary (BBC) endothelial cells were cultured as previously described (26) and used at passages 10–16. Cultures were maintained at 37°C, 5% CO_2 and 100% humidity.

Western blot analysis of HARP. The presence of HARP in the cell culture medium and in the extracellular compartments defined as cell surface and extracellular matrix, was investigated as previously described (5). The cells were allowed to grow to confluency in a 56 cm^2 tissue culture dish and the medium was changed to 2% fetal calf serum (FCS). Twenty-four hours later, the cells were washed twice with phosphate-buffered saline (PBS) pH 7.4 and subsequently with 20 mM Hepes pH 7.4, containing 2 M NaCl supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and 1 μg/ml aprotinin. The 2 M NaCl washes diluted 1:4, as well as the conditioned medium of the cells were incubated overnight with 100 μl of heparin-agarose at 4°C with continuous agitation. The heparin-agarose was washed three times with 10 ml of 20 mM Hepes pH 7.4, 0.5 M NaCl and twice with 10 ml of 20 mM Hepes, pH 7.4. Bound proteins were eluted with 50 μl of Laemmli sample buffer under reducing conditions, fractionated on 15% SDS-PAGE and transferred to Immobilon P membranes. Blocking was performed by air-drying the PVDF membranes, according to the manufacturer's instructions. The membranes were incubated with 1 μg/ml affinity purified HARP antibody in Tris-buffer saline (TBS), 0.2% (v/v) Tween-20 for 1 h at room temperature and then with horseradish peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:5.000. Detection of HARP was performed by ECL, according to the manufacturer's instructions.

Cell proliferation assays. The growth-promoting activity of HARP and its COOH and NH_2 peptides was determined by measuring the number of cells, using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay (33). HUVEC and RAME cells were seeded at 2×10^4 cells/well and BBC cells at 10^4 cells/well in 48-well tissue culture plates in the corresponding culture medium supplemented with 2% FCS. HARP or the peptides were added to the medium of the cells at concentrations ranging from 0.001 to 1 μg/ml and the number of cells was measured after 48 h. In another set of experiments, the 48-well tissue culture plates had been previously coated with 0.2 ml of solution of HARP or peptides in carbonate buffer at concentrations ranging from 0.001 to 1 μg/well. Cells were seeded at the same density as above, in the corresponding culture medium supplemented with 2% FCS and their number was determined 48 h later. 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 all wells and agitated thoroughly to solubilize the dark blue formazan crystals. The solution was transferred to a 96-well plate and immediately read on a microplate reader (Biorad) at a wavelength of 490 nm.

Statistical analysis. The significance of variability between the results from various groups was determined by one-way analysis of variance. Each experiment included triplicate wells for each condition tested. All results are expressed as mean $\pm$ SEM from at least three independent experiments.

RESULTS

Mitogenic Activity of HARP

We investigated the effect of human recombinant HARP expressed in bacterial cells as well as two peptides that correspond to the amino- and carboxyl-terminal parts of HARP on the proliferation of three endothelial cell types from three different sources, namely BBC, RAME and HUVEC. Since HARP is also present in the extracellular matrix of cells (2, 5), we also studied the mitogenic effect of HARP or the peptides when they were coated onto the cell culture plates.

HARP added in the cell culture medium of BBC cells had no effect on their proliferation even at a concentration higher than 100 ng/ml. In contrast, both the NH_2 and the COOH peptides of HARP used at concentrations between 1 and 1000 ng/ml induced a significant increase (about 80–100% maximum) of cell proliferation (Fig. 1A). Interestingly, a statistically significant effect (up to 70% over the control) was observed when HARP was coated onto the cell culture plate at a concentration higher than 300 ng/well (Fig. 1B). Both HARP peptides, when coated onto the

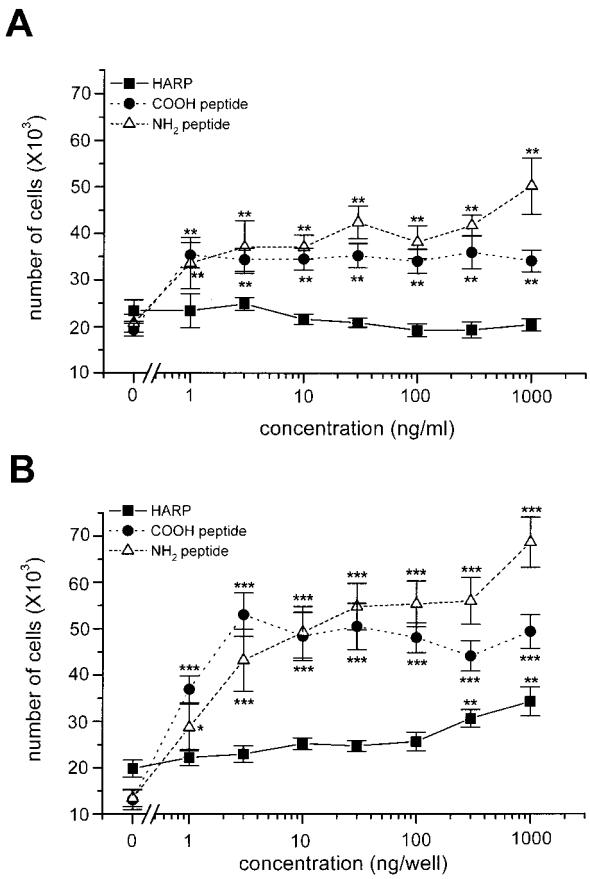

FIG. 1. Effect of human recombinant HARP expressed in bacterial cells and two peptides, corresponding to its COOH- and NH_2- termini, on the proliferation of BBC cells. HARP or peptides were added at different concentrations to the culture medium of the cells (A) or coated onto the corresponding wells of the cell-culture plates (B). Cells were further incubated for 48 h before measuring their number, as described under Materials and Methods. *, **, and *** denote statistical significance of $P < 0.05$, 0.01, and 0.001, respectively.

cell culture plate, also displayed a concentration-dependent mitogenic effect on BBC cells, which was much higher than the corresponding effect when the peptides were added as soluble molecule into the cell culture medium (Fig. 1). A highly significant effect was observed at a concentration lower than 3 ng/well for both peptides and the maximum increase of the number of cells was about 300% for the COOH peptide and 400% for the NH_2 peptide (Fig. 1B).

HARP, as well as both the NH_2 and the COOH peptides added in the cell culture medium of RAME cells, had no effect on their proliferation (Fig. 2A). In contrast, when the NH_2 or the COOH peptides were immobilized onto the cell culture plate, a concentration-dependent mitogenic effect was observed. The maximum increase, observed at 3 ng/well, was about 90% over the control for both peptides.

No effect on cellular proliferation was observed when HARP or the COOH peptide were presented as a soluble molecule in the cell culture medium of HUVEC. As shown in Fig. 3A, the NH_2 peptide had a small (about 30% over the control) and marginally statistically significant mitogenic effect on HUVEC. In contrast, when immobilized onto the cell culture plate, HARP had a concentration-dependent and statistically significant mitogenic effect on HUVEC (maximum 190% at a concentration of 10 ng/well). Both HARP peptides immobilized onto the cell culture plate displayed a concentration-dependent mitogenic effect (Fig. 3B). The maximum increase was observed at a concentration of 30 ng/well for both peptides and the stimulation was about 100% over the control.

Presence of HARP in the Extracellular Compartments and in the Culture Medium of Endothelial Cells

HARP is secreted by cells into the culture medium as well as sequestered in their extracellular compart-

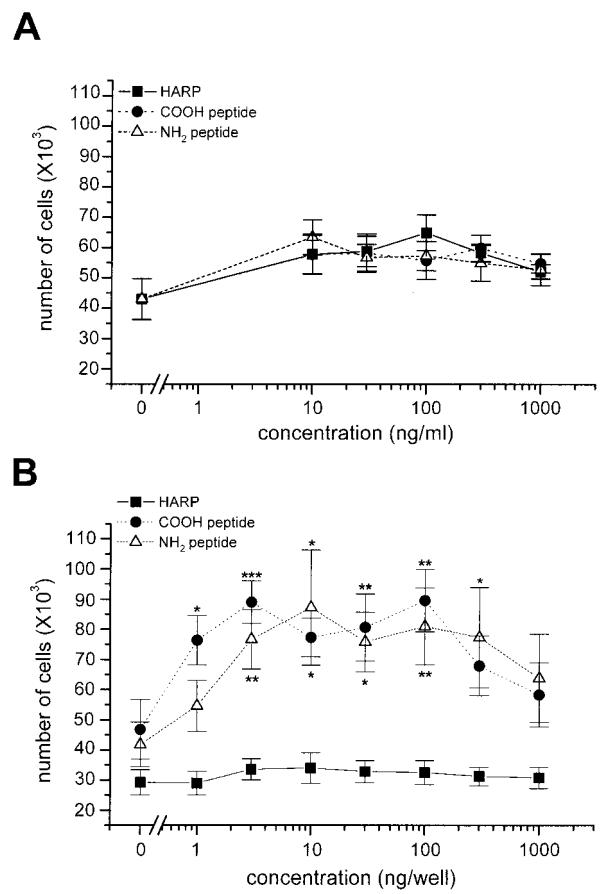

FIG. 2. Effect of human recombinant HARP expressed in bacterial cells and two peptides, corresponding to its COOH- and NH_2- termini, on the proliferation of RAME cells. HARP or peptides were added at different concentrations to the culture medium of the cells (A) or coated onto the corresponding wells of the cell-culture plates (B). Cells were further incubated for 48 h before measuring their number, as described under Materials and Methods. *, **, and *** denote statistical significance of $P < 0.05$, 0.01, and 0.001, respectively.

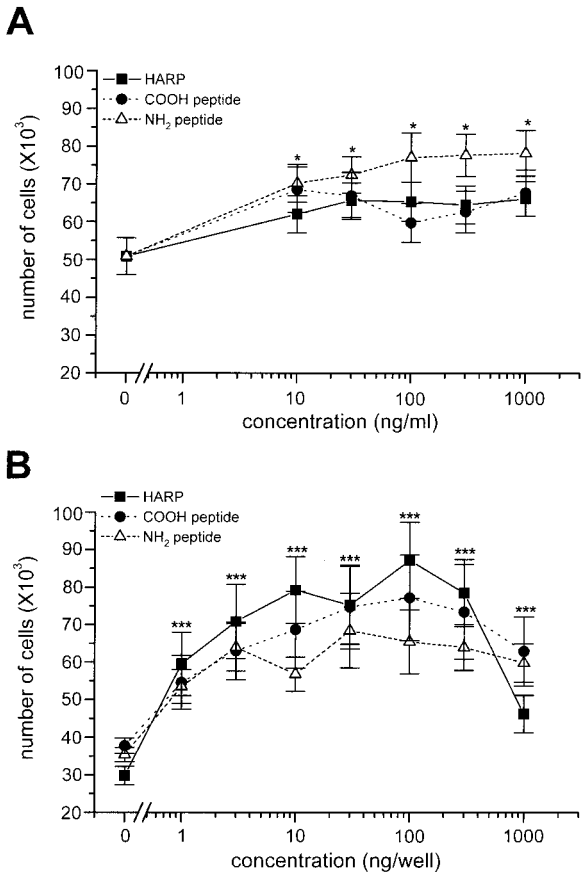

FIG. 3. Effect of human recombinant HARP expressed in bacterial cells and two peptides, corresponding to its COOH- and NH_2-termini, on the proliferation of HUVEC cells. HARP or peptides were added at different concentrations to the culture medium of the cells (A) or coated onto the corresponding wells of the cell-culture plates (B). Cells were further incubated for 48 h before measuring their number, as described under Materials and Methods. * and *** denote statistical significance of $P < 0.05$ and 0.001, respectively.

ments including matrix and cell surface (4, 5). The difference in the response of the endothelial cell types to HARP and its related peptides led us to determine whether these cells secreted and/or deposited this molecule and to what extend. Western blot analysis showed that HARP was secreted in the culture medium of BBC cells (Fig. 4, lane 2), while it was not detectable in the culture medium of RAME cells (Fig. 4, lane 4) and HUVEC (Fig. 4, lane 6). HARP was also detected in the 2 M NaCl washes of the surface of BBC (Fig. 4, lane 1) and RAME cells (Fig. 4, lane 3), which correspond to the amounts of HARP bound to the extracellular compartments. No signal was detected in the extracellular compartments of HUVEC (Fig. 4, lane 5). BBC produced much higher amounts of HARP than RAME cells. Comparing the profiles of HARP secreted in the culture medium and deposited on the matrix of the cells, it was obvious that BBC secreted higher amounts of HARP than RAME cells, which only secreted a smaller than 18 kDa form recognized by the anti-HARP antibody. This form was also detected in the cell fraction of both BBC and RAME cells. Interestingly, high molecular HARP immunoreactivity (27 kDa, 36 kDa and 50 kDa) was detected in the culture medium of BBC and the extracellular compartment of both BBC and RAME cells.

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 the proliferation of three endothelial cell types. Whatever the endothelial cell type, HARP had no significant effect on endothelial cell proliferation when it was added in the cell culture medium. These data agree to the previously published studies (2, 12, 25, 30) according to which only HARP produced in eucaryotic expression systems has mitogenic activity (2, 12), while HARP produced in prokaryotic expression systems lacks or has limited mitogenic effect (25, 30), due possibly to incorrect folding or incomplete processing of the recombinant polypeptide. Interestingly, however, when HARP expressed in bacterial cells was immobilized onto the cell culture plate, a dose-dependent mitogenic effect was observed in two of the three endothelial cell types studied. HARP binds with high affinity to heparin and thus to glycosamino-

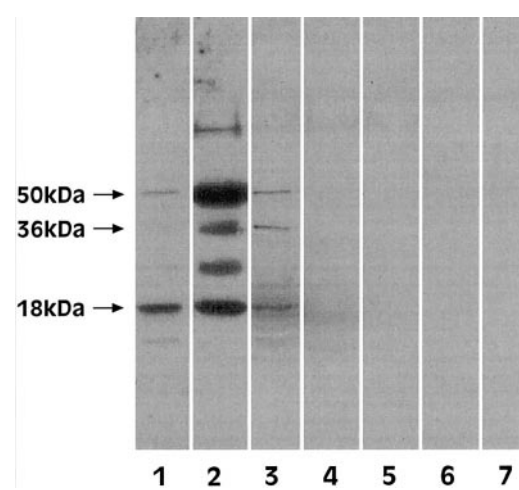

FIG. 4. Production and secretion of HARP by endothelial cells. (1) BBC cells and extracellular matrix bound material. (2) Conditioned medium of BBC cells. (3) RAME cells and extracellular matrix-bound material. (4) Conditioned medium of RAME cells. (5) HUVEC and extracellular matrix bound material. (6) Conditioned medium of HUVEC. (7) DMEM supplemented with 2% FCS. Conditioned media and the NaCl washes of the cells were collected as described under Materials and Methods and were incubated with 100 μl of a 10% suspension of heparin-agarose. Bound protein was eluted with Laemmli sample buffer and analyzed by SDS–PAGE followed by Western blot analysis, using an affinity-purified anti-HARP antibody.

glycans, including heparan sulfate (5, 35–37) and dermatan sulfate (5) derived from extracellular matrix, functioning possibly as a mitogenic molecule trapped in the extracellular space. A similar stimulating effect of HARP has been reported also for its *in vitro* neurite outgrowth activity. In that work, HARP was more effective when presented to chick embryo cerebral cortical derived neurons as a substrate than as a soluble factor (7). Endothelial cells exhibit polarized secretion of extracellular matrix molecules (32, 37) and localized matrix assembly sites (37). It is thus possible that the molecules on the endothelial cell surface involved in the binding and the transmission of HARP mitogenic signal are localized preferably on the basolateral side rather than the apical surface of the cells. A recent publication suggested that the binding of HARP to heparin was much tighter when HARP was immobilized on a surface (36). Thus, an alternative explanation to our results is that when HARP is immobilized onto the cell culture plate, it binds tighter to the cell surface glycosaminoglycans and thus exerts its mitogenic effect better than when it is presented in soluble form in the cell culture medium.

From the present study, it is not clear whether the mitogenic effect of HARP is due to interactions with cell surface proteoglycans or binding to another cell surface receptor. HARP receptors have been reported only for its neurite outgrowth activity. N-syndecan (syndecan-3), which mediates the neurite-promoting signal, is a transmembrane heparin sulfate proteoglycan that also binds other heparin-binding growth factors, like basic fibroblast growth factor (35). Another putative receptor for HARP is 6B4 proteoglycan/phosphacan, a truncated form of a receptor protein tyrosine phosphatase (RPTP)β/ζ (39). No specific cell surface macromolecules related to HARP mitogenic activity have been yet identified. Several studies have reported the existence of a high affinity binding site for HARP in several cell types including NIH 3T3 cells (24, 40) and bovine epithelial lens cells (41), where HARP action requires the activation of tyrosine kinase of the mitogen-activated protein kinase and PI3-kinase pathways (41). Heparitinase treatment of cells reduced HARP-induced cellular proliferation (5, 24) and glycosaminoglycans induced HARP dimerization, a key event in the activation of the transmembrane signaling receptors of several growth factors (34). It is possible that HARP interaction with cells may involve both glycosaminoglycans and a transmembrane specific receptor although it is not clear to what extent these interactions are involved in the mitogenic effect induced by HARP. Moreover, the diverse response of the different studied cells indicates that such interactions can vary among the cells, fact that renders the phenomenon even more complicated.

Interestingly, both peptides (HARP residues 1–21 and 121–139), had a concentration-dependent mitogenic effect, which was much greater when the peptides were immobilized onto the cell culture plate. It seems that the two lysine-rich terminal domains of HARP are implicated in multiple biological actions, since they stimulate endothelial cell proliferation (this paper) and are required for successful transformation of NIH 3T3 cells by HARP residues 41–64 (42). Both terminal regions account for the ability of HARP to bind tightly to heparin and to extracellular matrix (2, 4) and either of them was required for transformation of NIH 3T3 cells by HARP residues 41–64 (42), indicating that these domains have a similar functional role. This is also the case in our results, where the two peptides always had similar effect on endothelial cell proliferation. It has been hypothesized that the positively charged NH_2 and COOH-termini of HARP interact with the receptor or other molecules on the cell surface and facilitate HARP binding to a high affinity receptor (42). The existence of a high affinity HARP receptor, similar to the receptors of other heparin binding growth factors, has been strengthened by the finding that HARP dimerizes in the presence of glycosaminoglycans (34). In the present study, both HARP peptides had by themselves a significant effect on endothelial cell proliferation, which in the case of BBC and RAME cells was even higher than the effect of HARP itself. These results suggest that the peptides themselves bind to and activate the transmission of signals inside the cells. When both peptides were introduced simultaneously to the cells, the mitogenic effect was similar to that induced by each peptide alone, even at concentrations that did not induce maximal effect of each (data not shown). It seems that both peptides interact with the same molecule on the cell surface to induce endothelial cell proliferation with similar mechanism. Whether these or similar peptides exist physiologically, e.g. after proteolysis of HARP under certain circumstances, is not known at present and is under investigation.

It is not clear from our data whether the mitogenic effect of HARP or its peptides is due to an indirect mechanism involving displacement of other matrix-bound growth factors or to the interactions with specific cell surface receptor(s). Several studies have characterized the interaction of highly basic peptide including the exon 6-encoded sequence of VEGF (43) and the exon 6 of PDGF A chain (44) with cell surface glycosaminoglycans. These synthetic peptides also prevented the binding and the mitogenic activity of exogenously added growth factors like FGF-2 or PDGF-AA (45). In addition, it has been shown that the exon 6-encoded sequence of VEGF released iodinated FGF-2 bound to endothelial cells surface and was able to induce an angiogenic response in a corneal pocket assay, which was totally blocked by addition of anti-FGF-2 antibodies (43). These observations demonstrated that basic peptides such as axon 6-encoded sequence of

VEGF exert its mitogenic activity through FGF-2 signaling pathways. Due to their highly basic sequence, the HARP residues 1–21 and HARP residues 121–139 might be acting through an indirect mechanism involving de-sequestration of other matrix-bound growth factors.

The cells most responsive to HARP were BBC, which were those that expressed much higher amounts of HARP than the other studied cells. These cells derive from brain, where HARP has a functional role (11) and it is also possible that it is important for the growth of these cells as well. Interestingly, we have transfected BBC cells with anti-sense HARP mRNA but none of the transfections was successful because of cell death (unpublished observation). Control transfection with HARP sense mRNA continuously gave alive transfected cells. Another explanation could be that different cell surface molecules interact with HARP among the different cells, as discussed above.

In conclusion, human recombinant HARP expressed in bacterial cells had no mitogenic effect when presented as a soluble factor, while it is mitogenic when presented to endothelial cells as a substrate. HARP residues 1–21 and 121–139 seem to be involved in the mitogenic activity of HARP, suggesting that minimal structures could be sufficient to trigger endothelial cell proliferation if they are suitably presented to the cell. Interestingly, the effect of HARP and both peptides was quantitatively different among the different endothelial cells tested.

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