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Heparin affin regulatory peptide/pleiotrophin negatively affects diverse biological activities in C6 glioma cells

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Abstract

Heparin affin regulatory peptide (HARP) or pleiotrophin seems to be involved in the progression of several tumors of diverse origin. In this study, we tried to determine the role of HARP in rat C6 glioma cells by using an antisense strategy for inhibition of HARP expression. Decrease of the expression of endogenous HARP in C6 cells (AS-C6 cells) significantly increased proliferation, migration, and anchorage-independent growth of cells. Implantation of AS-C6 cells onto chicken embryo chorioallantoic membranes resulted in a significant increase of tumor-induced angiogenesis compared with that induced by non-transfected or C6 cells transfected with the plasmid alone (PC-C6 cells). In the same line, conditioned medium from AS-C6 cells significantly increased endothelial cell proliferation, migration, and tube formation in vitro compared with the effect of conditioned medium from C6 or PC-C6 cells. Interestingly, vascular endothelial growth factor (VEGF) induced C6 cell proliferation and migration, and SU1496, a selective inhibitor of VEGF receptor 2 (VEGFR2), blocked increased glioma cell growth, migration, and angiogenicity observed in AS-C6 cell cultures. The above results seem to be due to a direct interaction between HARP and VEGF in the culture medium of C6 and PC-C6 cells, while AS-C6 cells secreted comparable amounts of VEGF that do not interact with HARP. Collectively, these data suggest that HARP negatively affects diverse biological activities in C6 glioma cells, mainly due to binding of HARP to VEGF, which may sequester secreted VEGF from signalling through VEGFR2.

Keywords: Angiogenesis; Endothelial cells; Glioblastoma; Glioma cells; Heparin affin regulatory peptide; Pleiotrophin; Tumor; Vascular endothelial growth factor

Introduction

Heparin affin regulatory peptide (HARP), also known as pleiotrophin or heparin-binding growth-associated molecule, is an 18-kDa secreted growth factor that has high affinity for heparin. HARP is mainly expressed during embryonic development, as well as in early postnatal rat and bovine brain and was first isolated as a major neurite outgrowth-promoting protein of developing brain. Since then, a number of biological activities have been well established for HARP, such as its role in cellular proliferation, migration, differentiation and its involvement in bone formation, chondrogenesis, spermatogenesis, tumor growth, and angiogenesis. HARP is expressed in various cancer cell lines, derived from

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meningiomas, neuroblastomas, astrocytomas, melanomas, small cell lung cancer cell lines, and glioblastomas. Clinical studies have shown elevated serum levels and tumor expression of HARP in patients with colon, stomach, pancreatic, and breast cancer (reviewed in Papadimitriou et al., 2004; Mikelis et al., 2007).

Concerning the role of HARP in cancer, the majority of publications up to date suggest that it is a tumor-promoting factor. This notion is supported by data showing that overexpression of HARP in NIH 3T3 cells results in increased proliferation, anchorage-independent growth, and tumor formation in nude mice (Chauhan et al., 1993). In the same line, inhibition of HARP expression in cancer cells from breast, pancreas, prostate, or melanomas results in decreased cancer cell growth, colony formation, angiogenicity, and tumor growth in mice (Czubayko et al., 1996; Hatziapostolou et al., 2005; Weber et al., 2000; Zhang et al., 1997). On the other hand, it has also been suggested that overexpression of HARP in NIH 3T3 cells may be implicated in cellular quiescence rather than an oncogenic phenotype (Corbley, 1997). HARP has also been identified as a confluence-specific protein (Corbley, 1997; Merenmies, 1992; Papadimitriou et al., 2004) secreted by normal cells but not cells transformed by ras or other oncogenes (Corbley, 1997). In the same line, high HARP expression has been associated with poor vasculature in neuroblastomas and thus, better prognosis (Calvet et al., 2006), maybe due to a direct interaction of HARP with vascular endothelial growth factor (VEGF) (Heroult et al., 2004). Moreover, HARP expression is directly regulated by the transcription factor HOXA5, which induces apoptosis of breast cancer cells (Chen et al., 2005) and inhibits angiogenesis (Rhoads et al., 2005).

In the present work, we studied the functional significance of HARP in the growth and angiogenicity of C6 glioma cells, which are used as an established model for malignant glioma (Millauer et al., 1994; Saleh et al., 1996). Our data suggest that in C6 cells, HARP has an inhibitory effect on proliferation, migration, and growth in soft agar, paracrine effects of C6 cells on endothelial cell proliferation, migration and tube formation, as well as inhibition of angiogenesis in vivo, and this inhibitory effect is likely due to the binding of HARP to VEGF, which may sequester secreted VEGF from signalling through VEGF receptor 2 (VEGFR2).

Materials and methods

Human recombinant HARP was induced in *Escherichia coli* BL21 pLys cells transformed with the human HARP-pETHH8 plasmid and was purified by sequential heparin-sepharose and Mono-S chromatographies from bacteria, as previously described (Papadimitriou et al., 2000). Antibodies against VEGF and HARP were purchased from Santa Cruz Biotechnology, Inc. All secondary antibodies, protein A-agarose, SU1498, and all other reagents were purchased from Sigma. SU1498 was used at a concentration (5 μM) that was not toxic to cells.

Cell culture and immunofluorescence microscopy

C6 glioma cells (ATCC) were grown routinely in Ham's F12 medium supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 2.5 μg/ml amphotericin B. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords and cultured as previously described (Papadimitriou et al., 2000). HUVEC were grown as monolayers in medium M199 that was supplemented with 15% FBS, 150 μg/ml endothelial cell growth supplement, 5 U/ml heparin sodium, 100 U/ml penicillin–streptomycin, 50 μg/ml gentamycin, and 2.5 μg/ml amphotericin B and used at passage 2.

C6 glioma cells grown in 4-well tissue culture slides (Nunc) were fixed in 3% formaldehyde solution in phosphate-buffered saline (PBS), pH 7.4, for 30 min at room temperature, incubated with 0.5% Triton X-100 in PBS for 15 min at room temperature, blocked in PBS containing 3% bovine serum albumin (BSA) for 1 h at room temperature, and incubated with FITC-labelled phalloidin (0.5 μg/ml) in PBS for 1 h at room temperature in the dark. All incubations were performed under continuous agitation. Cells were photographed using a digital camera-equipped Zeiss Axioplan fluorescence microscope.

Stable transfection of C6 glioma cells

Full-length cDNA for HARP in antisense orientation (AS-HARP) was introduced in pCDNA3.1 vector, as previously described (Hatziapostolou et al., 2005). C6 glioma cells were seeded at 1×10^6 cells in 10-cm diameter cell culture Petri dishes in Ham's F12 containing 10% FBS and antibiotics. When confluency of cells reached 80%, transfection was performed using pCDNA3.1 alone or pCDNA3.1 carrying AS-HARP and poly-L-ornithine. Briefly, 10 μg of pCDNA3.1 or AS-HARP construct were diluted in 10 ml Ham's F12 medium containing 10% FBS and 10 μg/ml poly-L-ornithine. The mixture was added to the cells and the cells were incubated at 37 °C for 6 h. Cells were washed once with PBS and 10 ml of DMSO 30% (v/v) in medium was added to the cells for exactly 4 min at room temperature. Cells were then washed with PBS twice and fresh medium was added. Isolation of stable transfectants was performed by selection in 400 μg/ml neomycin