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Research paper

Proliferation and migration activities of fibroblast growth factor-2 in endothelial cells are modulated by its direct interaction with heparin affin regulatory peptide

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ABSTRACT

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. In normal or pathological angiogenesis, angiogenic growth factors activate cognate receptors on endothelial cells. Fibroblast growth factor-2 (FGF-2) and heparin affin regulatory peptide (HARP) are two heparin-binding growth factors and were described for their pro-angiogenic properties on human umbilical endothelial cells (HUVEC). We now show that HARP acts as a mediator of FGF-2's stimulatory effects, since it is able to inhibit the proliferation and migration of HUVEC induced by FGF-2. We demonstrate by ELISA and optical biosensor binding assay that HARP and FGF-2 interact through direct binding. We have adapted a previously developed structural proteomics method for the identification of residues involved in protein–protein interactions. Application of this method showed that two sequences in HARP were involved in binding FGF-2. One was in the C-thrombospondin type 1 repeat (C-TSR-1) domain and the other in the C-terminal domain of HARP. The identification of these regions as mediating the binding of FGF-2 was confirmed by ELISA using synthetic peptides, which are as well mediators of FGF-2-induced proliferation, migration and tubes formation on HUVEC *in vitro*. These results imply that besides a regulation of the proliferation, migration and angiogenesis of HUVEC by direct interaction of FGF-2 with its receptors, an alternative pathway exists involving its binding to growth factors such as HARP.

1. Introduction

Angiogenesis plays a key role in physiological and physiopathological processes. The formation of new capillaries from the endothelium of existing vasculature involves different steps, including degradation of the basement membrane, migration and proliferation of endothelial cells and stabilization of the new vascular tubes [1]. This process is tightly regulated by the balance between different pro and anti-angiogenic molecules, including growth factors. Among the pro-angiogenic growth factors, vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) represent the most important and extensively studied.

Abbreviations: a.a., amino acid; ALK, anaplastic lymphoma kinase; ASP, aspartic acid; CTGF, connective tissue growth factor; FGF-2, fibroblast growth factor-2; GLU, glutamic acid; HARP, heparin affin regulatory peptide; HBGF, heparin binding growth factors; HS, heparan sulfates; HUVEC, human umbilical vein endothelial cells; MK, midkine; RPTP β/ζ, receptor-type protein tyrosine phosphatase beta/zeta; $VEGF_{165}$, vascular endothelial growth factor 165; TSR-1, thrombospondin type 1 repeat.

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These two polypeptides are particularly implicated in normal and tumor angiogenesis [2,3].

Heparin affin regulatory peptide (HARP), also called pleiotrophin, is a 136 amino acid secreted polypeptide that forms with midkine (MK) a two-member family of heparin-binding growth factors (HBGF). Initially reported as a neurite outgrowth promoting molecule, intensive research on its ability to stimulate cell growth showed controversial results. Indeed, it appeared that HARP produced in mammalian system is mitogenic whereas HARP produced in bacteria is not [4]. In support of its role in angiogenesis, HARP has also been found to induce the migration of endothelial cells *in vitro* and *in vivo* [5,6]. HARP is a developmentally regulated cytokine that is highly expressed in the embryonic nervous system, but its expression drops dramatically after the perinatal phase and during adulthood. However, HARP has been shown to be overexpressed in various tumor cell lines and primary human tumors [7–9]. Two transmembrane proteins with intracellular catalytic domains have been described as HARP receptors: the receptor-type protein tyrosine phosphatase beta/zeta (RPTP β/ζ) and the anaplastic lymphoma kinase (ALK) receptor. The mitogenic and anti-apoptotic activities of HARP were initially linked to the high-affinity tyrosine kinase receptor ALK [10,11], whereas the neurite outgrowth, cell migration and adhesion activities of HARP were associated with the chondroitin sulfate proteoglycan RPTP β/ζ [12,13]. Structure-function studies have revealed that different regions of HARP are implicated in its biological activities. Thus, the C-terminal tail of HARP was reported to be implicated in the mitogenic, angiogenic and transforming activity of the protein [14,15]. The central region of HARP is composed of two β-sheet domains containing one thrombospondin type 1 repeat (TSR-1) motif each. These domains are linked by a flexible linker and maintained by disulfide bonds. The β-sheet domains were reported to be responsible for the binding of HARP to heparin and to its dimerization [16]. Interestingly, the β-sheet domains of HARP were also demonstrated to be responsible for the direct binding of HARP to $VEGF_{165}$. This direct binding inhibited the interaction of $VEGF_{165}$ to its receptors on endothelial cells causing a down-regulation of its angiogenic activity [17]. This inhibitory effect of HARP on $VEGF_{165}$, observed both *in vitro* and *in vivo*, suggested a dual regulatory function of HARP as both an angiogenic and angiostatic molecule.

In view of the foregoing, we hypothesized that HARP might also bind directly to FGF-2, which also plays an important role during angiogenesis, and interfere with its biological activity. The present study determined that HARP inhibited proliferation, migration and tubes formation of human umbilical vein endothelial cells (HUVEC) induced by FGF-2. Interaction between the two growth factors was highlighted by ELISA and optical biosensor binding assays. Moreover, we adapted a new approach to identify two main sequences of HARP interacting with FGF-2. So it appeared that the activities of FGF-2 can be modulated by its binding to HARP.

2. Materials and methods

2.1. Materials

Recombinant fibroblast growth factor-2 (FGF-2) was purified in the laboratory by sequential heparin Sepharose and Mono-S chromatography from bacteria [18] or by sequential heparin Sepharose and heparin HPLC affinity chromatography [19]. Recombinant Heparin Affin Regulatory Peptide (HARP) of bacterial origin was produced and purified in the laboratory as previously described [20]. Peptides P13–39 (SDCGEWQWSVCVPTSGDCGLGTREGTR) derived from the N-TSR-1 domain, P65–97 (AECKYQFQAWGECDLNTALKTRTGSLKRALHNA) derived from the C-TSR-1 domain and P111–136 (KLTKPKPQAESKKKKKEGKKQEKMLD) from the C-terminal tail of HARP were purchased from Altergen (Schiltigheim, France). Human umbilical vein endothelial cells (HUVEC), EGM-2 BulletKit medium were purchased from Lonza (Emerainville, France). Polyclonal antibodies against HARP and FGF-2 were from R&D Systems (France).

2.2. Proliferation and migration assays

HUVEC were routinely cultured in EBM-2 medium supplemented with 2% (v/v) fetal bovine serum (FBS) and BulletKit. Cultures were grown at 37 °C in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2. All experiments were carried out between passages 2 and 5. For cell proliferation assays, HUVEC were seeded into twelve-well plates (10 000 cells/well) in EBM-2 supplemented with 2% (v/v) FBS in the presence or absence of FGF-2 and HARP, P65–97, or P111–136. The cells were counted on day 5. For cell migration assays, HUVEC were seeded into a 24-well chemotaxis chamber (Transwell, Corning Costar, France). Polycarbonate filters of 8 μm pore size were coated with 10 μg/ml type I collagen R (Serva, Heidelberg, Germany) for 1 h and dried under sterile air. The EBM-2 medium supplemented with 1% (v/v) FBS was then placed in the lower chamber, with or without FGF-2, HARP, and different concentrations of P65–97 or P111–136 which served as chemoattractant. Cells (100 000/well) suspended in EBM-2/1% (v/v) FBS were seeded in the upper compartment. Transwells were incubated for 5 h at 37 °C, and treated as described previously [5].

2.3. ELISA binding assay

HARP or FGF-2 was coated at 1 μg/ml in 10 mM CAPS buffer and incubated overnight at 4 °C. For P111–136, P65–97 and P13–39 peptides, a range of concentrations was chosen for coating. After washing the wells with PBS containing 0.05% (v/v) Tween 20, non-specific binding sites were blocked by adding PBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma–Aldrich), for 1 h at 37 °C. After a rinse step, binding of FGF-2 or HARP was achieved in PBS containing 1% (w/v) BSA for 2 h at room temperature. The bound protein was characterized using 0.25 μg/ml of polyclonal antibody targeting HARP or FGF-2 and incubated for 1 h at room temperature. A peroxidase-labelled polyclonal anti-IgG was used at a concentration of 80 ng/ml for 30 min at 37 °C. Peroxidase activity was detected using 3,3,5,5-tetramethylbenzidine dihydrochloride substrate according to the supplier's instructions. Absorbance was measured at 450 nm.

2.4. Optical biosensor binding assays

HARP was immobilized on aminosilane surfaces using bissulfosuccinimidyl suberate (BS^3) as the cross linker following the manufacturer's recommendations (NeoSensors, Sedgefield, UK). No more than 700 arc *s* HARP was immobilized on the surface (1 arc $s = 1/3600°$, 600 arc $s = 1$ ng protein bound per mm^2). Binding assays were carried out in PBS supplemented with 0.02% (v/v) Tween 20 (PBST) at 20 °C following previously described methods [21–23].

2.5. Data analysis

To avoid artifactual second phase binding sites [24], assays were designed as described previously [25].

2.6. Protect and Label procedure

The method was adapted from the one described by Ori et al. [26], which was developed to identify binding sites in proteins for heparin. FGF-2 was biotinylated on its thiol residues using maleimide-PEG2-Biotin, No-Weigh™ Format (Pierce), as indicated by