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The hyaluronan-binding protease upregulates ERK1/2 and PI3K/Akt signalling pathways in fibroblasts and stimulates cell proliferation and migration

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Abstract

The hyaluronan-binding protease (HABP) is a serine protease in human plasma which is structurally related to plasminogen activators, coagulation factor XII and hepathocyte growth factor activator. It can in vitro activate the coagulation factor FVII, kininogen and plasminogen activators. The present study was initiated to gain a more complete picture of the cell-associated activities of this fibrinolysis-related protease. Treatment of lung fibroblasts with HABP lead to a rapid activation of signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway with c-Raf, MEK and ERK1/2. Additionally the activation of the PI3K/Akt pathway and of several translation-related proteins was found. Proliferation assays confirmed the assumption of a strong growth-stimulating effect of HABP on human lung and skin fibroblasts. Intracellular signalling and growth stimulation were strongly dependent on the proteolytic activity of HABP. Stimulation of signalling and proliferation by HABP involved the fibroblast growth factor receptor 1 (FGFR-1). HABP-stimulated proliferation of lung fibroblasts MRC-5 was accompanied by a significant intracellular increase in basic fibroblast growth factor (bFGF), the major ligand of FGFR-1; bFGF could however not be identified in the supernatant of HABP-treated cells. Though, the conditioned medium from HABP-treated cells showed a strong growth-promoting activity on quiescent fibroblasts, indicating the release of a yet unknown growth factor amplifying the initial growth stimulus. In a two-dimensional wound model HABP stimulated the invasion of fibroblasts into a scratch area, adding a strong pro-migratory activity to this plasma protease. In summary, HABP exhibits a significant growth factor-like activity on quiescent human lung and dermal fibroblasts. Our findings suggest that this fibrinolysis-related plasma protease may participate in physiologic or pathologic processes where cell proliferation and migration are pivotal, like tissue repair, vascular remodelling, wound healing or tumor development.

Keywords: bFGF; FGFR-1; HABP; ERK1/2; MRC-5; PI3/Akt; Skin fibroblasts

1. Introduction

A novel proteolytic activity was identified in human plasma during fractionation of vitamin K dependent coagulation factors and was purified to homogeneity [1]. The 65 kDa two-chain enzyme represented the active form of a plasma hyaluronan-binding protein [2], termed hyaluronan-binding protease. This enzyme was independently described as a coagulation factor VII and plasminogen activator-activating protease [3,4] thus attributing procoagulatory and profibrinolytic activities to HABP. Other in vitro studies indicated that HABP can release the vasoactive peptide bradykinin from kininogen [5], causing an intracellular calcium release in HUVECs via activation of the bradykinin B2-receptor [6]. HABP could also induce signalling pathways in HUVECs independent of bradykinin leading to the activation of the mitogen-activated protein kinase (MAPK) ERK1/2 and the transcription factors c-myc

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and CREB [6]. Despite a transient activation of the ERK1/2 pathway in quiescent cells, bFGF/EGF-stimulated HUVEC proliferation was inhibited by HABP, due to specific inactivation of the proangiogenic basic fibroblast growth factor (bFGF) [7]. Interestingly, the platelet-derived growth factor BB (PDGF-BB)-mediated proliferation and migration of vascular smooth muscle cells by HABP was also inhibited as a result of neutralisation of the growth factor [8].

The high structural similarity of HABP to the plasminogen activator urokinase (uPA) [9] raised the hypothesis of urokinase-like activities on a cellular level. The plasminogen activator/plasmin system on the cell surface participates in matrix degradation and facilitates cell migration [10]. Additionally, uPA binds to its receptor (uPAR, CD87), which in turn can cause cytoskeletal changes, the activation of kinases and cell migration [11]. Cell proliferation and migration are important processes in wound healing, in development, angiogenesis and tumor metastasis.

Basic FGF has been proposed as a factor promoting wound healing, regeneration and revascularisation of tissues [12]. It is expressed by a variety of cells, among those endothelial cells and fibroblasts [13]. Fibroblasts are critical in many aspects of tissue repair and remodelling. During wound healing fibroblasts synthesize and deposit the extracellular matrix (ECM), a process that is critical for the recruitment of new fibroblasts and for the migration of endothelial cells to form new vessels. Upon migration into a wounded site, fibroblasts become profibrotic, produce a collagen-type ECM and acquire a contractile phenotype that facilitates closure of the wound [14].

In order to gain a broader insight in the cell-directed functions of this plasma protease, our studies were extended to cell types that release growth factors in autocrine and paracrine mode and are important in tissue regeneration and wound repair. Here we demonstrate for the first time that the hyaluronan-binding protease HABP is not only able to inhibit cell growth by growth factor inactivation as observed earlier, but exhibits also significant growth factor-like activity on quiescent lung and skin fibroblasts, upregulating intracellular signalling pathways and stimulating cell proliferation and migration. The physiologic and pathologic relevance of these findings is discussed.

2. Material and methods

HABP was prepared as described earlier [1]. MTT reagent 3-[4,5 dimethyldiazol-2]-2,5-diphenyl tetrazolium bromide, calcein-AM, Wortmannin, gelatine (2%) and bovine serum albumin (BSA) were purchased from Sigma (Deisenhofen, Germany). All plastic equipment used in cell culture was from NUNC (Wiesbaden, Germany). Unless stated otherwise the antibodies and inhibitors used for the study of intracellular signalling were from Cell Signaling/New England BioLabs (Frankfurt/Main, Germany). All other reagents were obtained from Gibco/Invitrogen (Karlsruhe, Germany) or Merck Eurolab (Darmstadt, Germany).

2.1. Cell culture

Unless stated otherwise cell lines were cultured under the following conditions at 37 °C in a humidified 5% CO_2 atmosphere. Human lung fibroblasts (MRC-5, in-house culture) [15] or human skin fibroblasts (HSFB) isolated from skin biopsies from 10 donors (Technoclone, Vienna, Austria) were cultured to confluence in uncoated 80 cm^2 tissue culture flask in CMRL 1969 supplemented with 5% FCS, 2.2 g/l $NaHCO_3$, 10 mM L-glutamine, 10 μg/ml streptomycin, 10 U/ml penicillin. MRC-5 lung fibroblasts from passages 29 to 41 and HSFB from passages 6 to 8 were used. For serum-free conditions CMRL 1969 supplemented with 2.2 g/l $NaHCO_3$, 10 mM L-glutamine was used.

2.2. Cell proliferation assay

First, in the absence of HABP cells were allowed to attach to the uncoated plates for 5 h in CMRL 1969 supplemented with 5% FCS, 2.2 g/l $NaHCO_3$, 10 mM L-glutamine, 10 μg/ml streptomycin, 10 U/ml penicillin. Subsequently cells were cultured in CMRL 1969 under serum-free conditions for 4 days in the presence of different concentrations of HABP. Finally, adherent viable cells were quantified by the MTT assay or the number of adherent cells was counted in a Neubauer chamber.

2.3. MTT assay

This assay is based on the catalytic activity of cellular oxidoreductases like mitochondrial dehydrogenases in living cells, resulting in the cleavage of the substrate MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] which forms water-unsoluble formazan crystals. A final concentration of 0.5 mg/ml MTT reagent was added directly to the cell supernatant. After 2.5 h the cell culture medium was removed by decanting. Residual supernatant was removed by tapping off suction on paper towels and the formazan crystals in adherent cells were dissolved in ethanol/DMSO (1 : 1 v/v). The absorbance of the formazan solution was measured in 1 cm cuvettes at 570 nm in a spectrophotometer (Amersham-Pharmacia, Freiburg, Germany). A strong correlation (correlation coefficient = 0.98) with a slope of approximately 1 was obtained when the total number of adherent living MRC-5 cells was plotted versus the MTT signal intensity ($n = 105$ data points). A 100% increase in the MTT signal corresponds to approximately a doubling in cell number (MRC-5 cells). Thus this assay was considered suitable for quantitative analysis of the number of adherent living cells.