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Hyaluronectin modulation of lung metastasis in nude mice

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ABSTRACT

Hyaluronectin (HN) is a glycoprotein with a high affinity to hyaluronic acid (HA) and known to be a component of the extracellular matrix of tumours. Clinical studies have shown that a low level of HN correlates to tumours with poor prognosis, whereas a high level of HN correlates to tumours with good prognosis. We previously demonstrated *in vitro* that hyaluronidase activity, which promotes tumour progression and metastatic spread by degradation of HA into angiogenic oligosaccharides, was inhibited or promoted by HN, according to the level of HN-expression. This raises the question of the role played by HN in cancer, and particularly if high and low levels of HN-expression could trigger opposite effects on tumour growth and/or metastatic spread. To address this issue, we used a model of spontaneous lung fluorescent metastases that we characterised previously. We stably transfected the human HN cDNA into fluorescent H460M^{GFP} cells and selected two clones characterised by different levels of HN-expression: HN110 and HN704, with a high and a low level of HN-expression, respectively. *In vitro*, we demonstrated that HN704 cell migration was significantly increased. Inoculation of clones to nude mice had no significant effect on tumour growth, but clearly revealed opposite effects on metastatic spread: HN110 significantly decreased the number of fluorescent metastases whereas HN704 significantly increased it. We also analysed HN, HA and hyaluronidase contents in sera and tumours. These results demonstrate that HN can play a role as either a suppressor or promoter of metastatic spread.

1. Introduction

During tumour development, cancerous cells induce numerous changes in their microenvironment. The modifications of the extracellular matrix trigger a set of physiological responses such as development of new blood vessels (neo-angiogenesis), allowing tumour growth to carry on, or migration of cancerous cells towards blood vessels, leading to spread of metastases. Hyaluronic acid (HA), also known as hyaluronan, is a major component of the extracellular matrix, which is greatly implicated in several of these responses.[1] The degradation of this polysaccharide into small angiogenic fragments by hyaluronidases promotes tumour growth[2] and metastatic spread by triggering endothelial cell migration and capillary formation.[3–5] Hyaluronectin (HN), a glycoprotein discovered in 1979 in brain and in cancer tumour stroma,[6] exhibits a great affinity for HA.[7] The amino acid sequence of HN matches perfectly with that of the HA-binding domain of

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versican, a large proteoglycan.[8] HN/HA complexes dissociate at acidic pH, which allow purification of HN by affinity chromatography on insolubilised HA.[9,10] Studies made with antibodies directed against the HA-binding domain of versican located the versican HA-binding domain to the nodes of Ranvier,[11] a well-known location of HN.[12,13] The association of HN with cancer stroma led to the question of whether this protein could play a role in cancer development, likely as a modulator of HA. Indeed, in brain tumours, the HN/HA ratio is much lower in glioblastomas than in low-grade astrocytomas.[14] In breast cancer, we find that medullary tumours, whose prognosis is better than that of other breast adenocarcinomas, were characterised by HN-rich mononuclear infiltrates.[15] HN release by monocytes is modulated by interleukins.[16] *In vitro*, HN inhibits the promotion of endothelial cell migration by HA-derived oligosaccharides,[17] suggesting that HN can exert an anti-angiogenic effect *in vivo*. Moreover, HN is also able to inhibit *in vitro* hyaluronidase activity of cancer cells at pH 3.8 at concentrations above 100 μg/ml, whereas this activity is very efficiently enhanced at concentrations below 50 μg/ml.[18,19]

In the present work, we evaluated *in vivo* the impact of different levels of HN-expression on tumour growth and metastatic spread. We took advantage of a model of fluorescent spontaneous lung metastases that we developed in nude mice[20,21] to demonstrate that, according to the level of expression, HN could act as either a promoter or suppressor of metastatic spread. These results provide the first evidence for the implication of HN as a modulator of metastatic spread, and suggest a complex mode of action.

2. Materials and methods

2.1. Cell lines and culture

The H460M^{GFP} cell line was obtained and characterised as previously described.[20,21] This cell line was derived from the H460M cell line and stably expresses the cDNA of the Green Fluorescent Protein (GFP). All cell lines were maintained as adherent monolayers in RPMI-1640 medium (Life Technologies, Pontoise, France) supplemented with 10% foetal calf serum (Roche Diagnostic, Meylan, France) and 2 mM L-glutamine (Life Technologies, Pontoise, France) at 37 °C in a humidified incubator with 5% CO_2 in air. The cells were grown to 80% confluence, washed two times with phosphate buffered saline and then harvested after a brief treatment with trypsin/EDTA (Sigma, Saint-Quentin Fallavier, France). Cell viability was determined by trypan blue dye exclusion.

2.2. Construction of a recombinant expression vector containing the human HN cDNA

The pBlueScript vector (Stratagene) containing the cDNA of the HA-binding domain of versican[8] was kindly provided by Dr. Zimmerman (Department of Pathology, University of Zurich, Switzerland). This cDNA was inserted into the bicistronic mammalian expression vector pIRES-Hyg (Clontech, Palo Alto, USA), allowing the selection of mammalian cells by expression of hygromycin B resistance. The inserted sequence, cloned into *Bam*HI and *Bst*XI restriction sites of pIRES-Hyg vector, extends from nucleotides 1–1372 of the human versican N-terminus portion and corresponds to the whole human HN sequence. The final vector, used for transfections and encoding the human HN protein, was named pHN-IRES-Hyg.

2.3. Transfection and isolation of cellular clones expressing HN

The H460M^{GFP} cell line was used as the parental cell line to obtain HN-expressing cells. Transfections were performed in near confluent cells by the Fugene-6 procedure (Roche, Meylan, France). One day before transfection, cells were seeded at a density of 3×10^5 cells in 35-mm culture dishes (Corning Costar, Brumath, France). For transfection, 6 μl of Fugene-6 reagent were diluted in 94 μl of serum-free medium. The solution was incubated for 10 min with 2 μg of vector and then added to the cells. The cells were harvested by trypsin/EDTA for 24 h after transfection, and subcultured at a ratio of 1:10 in a selective medium that contained 200 μg/ml of Hygromycin B (Clontech). Ten to 14 days after selection, surviving cells were cloned by limiting dilution. Individual clones were expanded and screened for HN-expression.

2.4. Microscopy and cytofluorimetry

Microscopic evaluation of GFP-expression in cell lines was performed as previously described[20,21] by direct observation of cells using a fluorescence microscope (Axiovert 10, Zeiss) equipped with a double-pass filter set for FITC/PI (excitation filter BP450-490; suppression filter LP520). Cell lines fluorescence was analyzed by FACS (FACS Calibur, Becton Dickinson, Omaha, CA) with a standard excitation wavelength of 488 nm.

2.5. HN determination

HN was quantitated by ELISA using HA-coated (to test the affinity to HA) and antibody-coated plastic microplates as previously described;[9] both techniques gave similar results ($R^2 = 0.96$). The use of antibody-coated plates allowed digestion of the samples with bovine testicular hyaluronidase before the assay to establish that no HN would be masked by HA. Briefly, samples were diluted in 10 mM sodium phosphate buffer containing 1 M NaCl, 0.2 g/l EDTA, 0.25 g/l sodium azide and 1 g/l BSA, at pH 7.4 and incubated in plate wells for 3 h at 4 °C. HN bound to the plate was measured with alkaline phosphatase-linked rabbit antibodies to human HN, and staining was performed with *p*-nitrophenyl phosphate at pH 9.8.

2.6. Western blot analysis

HN detection was performed on concentrated serum-free culture medium. HN was precipitated from the culture medium by addition of saturated ammonium sulfate at 4 °C for 48 h. The precipitate was dissolved in PBS (1/10 of the initial volume), dialyzed against PBS and HN content was determined with the ELISA method. Twenty microlitre samples were electrophoresed in 0.2% SDS w/v on 15% polyacrylamide v/v gel with a 4% v/v stacking gel according to Laemmli[22] for 40 min (Hoeffer, Bioblock, Strasbourg, France). Electrophoretic