NEOPLASIA
www.neoplasia.com

Volume 14 Number 2 February 2012 pp. 95–107 95

Membrane-Bound and Exosomal Metastasis-Associated C4.4A Promotes Migration by Associating with the $\alpha_6\beta_4$ Integrin and MT1-MMP[1,2]

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Abstract

Metastasis-associated C4.4A, which becomes upregulated during wound healing and, in some tumors, during tumor progression, is known to be frequently associated with hypoxia. With the function of C4.4A still unknown, we explored the impact of hypoxia on C4.4A expression and functional activity. Metastatic rat and human tumor lines upregulate C4.4A expression when cultured in the presence of $CoCl_2$. Although hypoxia-inducible factor 1α (HIF-1α) becomes upregulated concomitantly, HIF-1α did not induce *C4.4A* transcription. Instead, hypoxia-induced C4.4A up-regulation promoted *in vivo* and *in vitro* wound healing, where increased migration on the C4.4A ligands laminin-111 and -332 was observed after a transient period of pronounced binding. Increased migration was accompanied by C4.4A associating with $\alpha_6\beta_4$, MT1-MMP1, and TACE and by laminin fragmentation. Hypoxia also promoted the release of C4.4A in exosomes and TACE-mediated C4.4A shedding. The association of C4.4A with $\alpha_6\beta_4$ and MT1-MMP1 was maintained in exosomes and exosomal $\alpha_6\beta_4$- and MT1-MMP1–associated C4.4A but not shed C4.4A sufficient for laminin degradation. Hypoxia-induced recruitment of $\alpha_6\beta_4$ toward raft-located C4.4A, MT1-MMP, and TACE allows for a shift from adhesion to motility, which is supported by laminin degradation. These findings provide the first explanation for the C4.4A contribution to wound healing and metastasis.

Neoplasia (2012) 14, 95–107

Introduction

C4.4A is a glycosyl-phosphatidyl-inositol–anchored molecule and belongs, like the urokinase-type plasminogen activator receptor (uPAR), to the Ly6 family [1–3]. C4.4A shares with uPAR three-finger protein domains, characterized by three to six *S-S* bridges, which guarantee maintenance of domain structure by stabilizing the hydrophobic nucleus of the protein [4,5]. uPAR has three and C4.4A two strongly hydrophobic three-finger protein domain [6]. C4.4A has 5 to 6 *N*-glycosylation sites close to the second TFP domain and 15 *O*-glycosylation sites in a Ser/Thr–rich region at the C-terminus [7]. C4.4A associates with laminins (LN) 111 and 332 (formerly LN1 and LN5) and galectin-3 [8].

C4.4A expression is restricted to the basal and suprabasal layers of squamous epithelium in nontransformed tissue [1,2,7,9,10], where it becomes upregulated during wound healing [7,10]. High C4.4A expression has been seen in several types of carcinoma like

Abbreviations: $\alpha_6\beta_4$, $\alpha_6\beta_4$ integrin; aprotinin, serine protease inhibitor; AS, BSp73AS; AS1B1, AS cells transfected with C4.4A cDNA; ASML, BSp73ASML; FN, fibronectin; HRE, HIF response element; LN, laminin; LN111, formerly LN1; LN332, formerly LN5; MMP14, membrane type 1 matrix metalloproteinase/MT1-MMP; MMP-Inh.II, MMP9/13-inhibitor-II; Prog, progressor; RPMI, RPMI-1640; TACE, ADAM17; TAPI, TACE inhibitor; WB, Western blot

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[1]This work was supported by the Deutsche Krebshilfe (10-1821-Zö3 to M.Z.). H. Ngora is a PhD grant recipient of the Deutscher Akademischer Austausch Dienst (German Academic Exchange Service). The authors declare no conflict of interest.

[2]This article refers to supplementary materials, which are designated by Tables W1 to W3 and are available online at www.neoplasia.com.

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Received 16 October 2011; Revised 28 November 2011; Accepted 29 November 2011

DOI 10.1593/neo.111450

mammary, renal cell, colorectal [11–13] and most pronounced non–small cell lung cancer [14]. Its expression in other types of cancer, like esophageal cancer becomes regulated during tumor progression [15,16]. C4.4A transcription requires C/EBPβ and is enhanced by JunD or c-Jun [17], which fits to upregulated expression during wound healing [18–22].

We here aimed to shed light on the molecular mechanism of C4.4A activities, which remain elusive, despite C4.4A being consistently associated with tumor progression and wound healing [3]. These two activities of C4.4A are frequently associated with hypoxia [23,24], which initiates a gene transcription program frequently through hypoxia-inducible factor (HIF) [25,26]. Stabilized HIF-1α undergoes nuclear translocation and associates with transcriptional coactivators [27,28]. Because the C4.4A promoter contains three potential HIF-1α response elements (HREs), we first evaluated whether hypoxia-induced HIF-1α may contribute to *C4.4A* transcription and whether hypoxia influences C4.4A activity in wound healing and tumor cell migration. Under hypoxia, C4.4A forms a complex with $\alpha_6\beta_4$ and MMP14 (formerly MT1-MMP), which promotes motility possibly through focalized LN332 degradation.

Materials and Methods

Tumor Lines

The rat tumor lines were BSp73ASML (ASML, C4.4A$^+$, $\alpha_6\beta_4^+$, metastasizing), BSp73AS (AS, C4.4$^-$, $\alpha_6\beta_4^-$, nonmetastasizing) [29], and BSp73AS1B1 (AS1B1, C4.4A cDNA-transfected AS clone, C4.4A$^+$, $\alpha_6\beta_4^-$). The coding sequence of the C4.4A cDNA has been cloned into the pcDNA3 vector with a CMV promoter to drive C4.4A transcription [1]; Progressor (Prog) (C4.4A$^+$ $\alpha_6\beta_4^+$) [30], 804G (LN332 secreting) [31], and the human A431 (LN332 secreting) [32] were maintained in RPMI/10% fetal calf serum (FCS). The human pancreatic cancer lines Capan-2 (metastasizing) [33], Colo357 (metastasizing) [34], 8.18 (weakly metastasizing) (Tumor Bank, German Cancer Research Center, Heidelberg, Germany; personal observations), and BxPC3 (nonmetastasizing) [35] were maintained in RPMI/10% FCS/10 mM Na-pyruvate. Confluent cultures were trypsinized and split. Where indicated, cells were treated with 100 to 200 μM $CoCl_2$ for 6 to 24 hours or maintained at 1% O_2 for 6 to 12 hours.

Antibodies, Matrix Proteins, and Inhibitors

Antibodies, matrix proteins, and inhibitors are listed in Table W1.

Vesicle Depletion and Exosome Preparation

Cells were cultured (48 hours) in serum-free medium. Cleared supernatants (2 × 10 minutes at 500*g*, 1 × 20 minutes at 2000*g*, 1 × 30 minutes at 10,000*g*) were centrifuged (90 minutes at 100,000*g*) and washed (phosphate-buffered saline, 90 minutes at 100,000*g*). The supernatant was collected as vesicle-depleted fraction. The pellet (crude exosomes) was suspended in 2.5 M sucrose, overlaid by a continuous sucrose gradient (0.25-2 M), and centrifuged (15 hours at 150,000*g*).

Flow Cytometry

Flow cytometry followed routine procedures. For intracellular staining, cells were fixed and permeabilized. For chloramphenicol acetyltransferase (CAT) assay standardization, cells were cotransfected with the EGFP-C1 plasmid to evaluate transfection efficacy. Cells were analyzed in a FACScan using the Cell Quest analysis program.

Reverse Transcription–Polymerase Chain Reaction

Total RNA preparation followed standard procedures. Primers are listed in Table W2.

C4.4A Promoter Constructs, Mutations, Transfection, and CAT Assay

A 588-bp and a 1.9-kbp C4.4A promoter constructs have been used [17]. Three CGTG HRE sequences of C4.4A at −673 bp (HRE1), −1183 bp (HRE2), and −1557 bp (HRE3) are shown in Table W3. C4.4A sequences were inserted in promoterless pBLCAT3-Basis (pBLCATB3), which contains the CAT gene [36]. Tk promoter-containing pBLCAT2 served as control [37]. Transfection (3 μg of pBLCAT3-C4.4A, 1 μg of EGFP-C1 or 1.75 μg of pBLCAT3-C4.4A, 1.25 μg of p(HA) HIF-1α 401Δ603 [HIF-1α with oxygen-dependent degradation domain deletion], 1 μg of EGFP-C1) was done as described [17]. For the CAT assay [38], lysates were standardized for protein content and transfection efficacy. Thin-layer chromatography was quantitated using BAS-1800II PhosphorImager (Fuji, Dusseldorf, Germany).

Immunoprecipitation, SDS-PAGE, and Western blot

Cell lysates (60 minutes, 4°C, HEPES buffer, 1% Brij96, protease inhibitor cocktail) were centrifuged (13,000*g* for 10 minutes at 4°C), incubated with antibody (overnight), and precipitated with ProteinG Sepharose (1 hour at 4°C). Washed immune complexes were dissolved in Laemmli buffer. Precipitates/lysates were resolved on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (30 V for 12 hours at 4°C); membranes were blocked, blotted with primary and HRP-conjugated secondary antibodies (1 hour at room temperature), and developed with the ECL kit or were stained with Coomassie blue.

Immunofluorescence and Immunohistochemistry

Cells seeded on bovine serum albumin (BSA)–, LN111-, LN332-, or fibronectin (FN)-coated cover slides were fixed; permeabilized; blocked; incubated with primary antibody (60 minutes at 4°C); fluorochrome-conjugated secondary antibody (60 minutes at 4°C); blocked, incubated with a second, dye-labeled primary antibody (60 minutes at 4°C); and washed. Where indicated, cells were removed by EDTA. Cover slides were mounted in Elvanol (Sigma Aldrich, Steinheim, Germany). Shock-frozen skin sections (7 μm) were exposed to primary antibody, biotinylated secondary antibody, and alkaline phosphatase-conjugated avidin-biotin complex solutions. Sections were counter stained with hematoxylin and eosin. Digitized images were generated using a Leica DMRBE microscope (Leica, Wetzlar, Germany), a SPOT CCD camera, and Software SPOT2.1.2 (Sterling Heights, MI).

Adhesion and Migration Assays

Adhesion to coated 96-well plates was determined after 30 and 240 minutes (37°C). Nonadherent cells were removed by washing. Migration was evaluated in Boyden chambers seeding cells in the upper chamber (RPMI/1% BSA) with/without $CoCl_2$ and/or protease inhibitors. The lower chamber, separated by an 8-μm pore size polycarbonate membrane, contained RPMI/1% BSA or LN332 (804G supernatant). In both assays, cells were stained with crystal violet, measuring OD_{595nm} after lysis. Adhesion/migration is presented as percentage input cells. For *in vitro* wound healing, a subconfluent