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Stroma-induced Jagged1 expression drives PC3 prostate cancer cell migration; disparate effects of RIP-generated proteolytic fragments on cell behaviour and Notch signaling

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The Notch ligand Jagged1 is subject to regulated intramembrane proteolysis (RIP) which yields a soluble ectodomain (sJag) and a soluble Jagged1 intracellular domain (JICD). The full-length Jagged1 protein enhances prostate cancer (PCa) cell proliferation and is highly expressed in metastatic cells. However, little is known regarding the mechanisms by which Jagged1 or its RIP-generated fragments might promote PCa bone metastasis. In the current study we show that bone marrow stroma (BMS) induces Jagged1 expression in bone metastatic prostate cancer PC3 cells and that this enhanced expression is mechanistically linked to the promotion of cell migration. We also show that RIP-generated Jagged1 fragments exert disparate effects on PC3 cell behaviour and Notch signaling. In conclusion, the expression of both the full-length ligand and its RIP-generated fragments must be considered in tandem when attempting to regulate Jagged1 as a possible PCa therapy.

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1. Introduction

The Notch signaling pathway has been linked to tumour development/progression with aberrations being tumour-suppressive or -oncogenic in a wide range of cancers [1]. The mammalian pathway is activated when a Notch receptor and ligand (Jagged1, Jagged2, Delta-like ligand 1 (Dll1), Dll3, or Dll4 [1]) on the surface of neighbouring cells interact, initiating regulated intramembrane proteolysis (RIP) of the receptor. This, ultimately, leads to the formation of a soluble Notch intracellular domain (NICD), a transcriptional regulator which translocates to the nucleus [1].

The Notch ligand Jagged1 is a 185 kDa membrane protein which, like its receptor, is subject to RIP [2,3]. The full-length protein is cleaved between E1054 and V1055 [4,5] by one or more members of the A Disintegrin And Metalloproteinase (ADAM) family of zinc metalloproteinases to liberate a soluble ectodomain (sJag) and a membrane-associated Jagged1 C-terminal fragment (JCTF). This latter fragment is subsequently cleaved by a γ-secretase activity to yield the soluble Jagged1 intracellular domain (JICD) [2]. The JICD is a functional transcriptional regulator like the NICD [2] and contains a putative nuclear localisation signal (nls) 'RKRRK' between R1094 and K1093.

A considerable body of work indicates an important role for Jagged1 in prostate cancer (PCa). Down-regulation of the protein in a range of PCa cells inhibits proliferation and induces growth arrest in the S phase of the cell cycle, possibly due to the reduced expression or activity of S phase-associated cyclins and cyclin-dependent kinases [6]. Jagged1 expression in PCa cells also increases the expression of anti-apoptotic proteins [7]. Expression of the ligand is significantly higher in metastatic PCa compared to benign or localized disease, further indicating that the protein may be linked to the growth, metastasis and progression of prostate tumours [8]. However, despite an unarguable role for Jagged1 in PCa cell proliferation and its circumstantial link with metastatic disease, nothing is known of the mechanisms by which the ligand

Abbreviations: ADAM, A disintegrin and metalloproteinase; BMECs, bone marrow endothelial cells; BMS, bone marrow stroma; Dll, Delta-like ligand; FL-Jag, full-length Jagged; JCTF, Jagged1 C-terminal fragment; JICD, Jagged1 intracellular domain; NICD, Notch intracellular domain; nls, nuclear localisation sequence; PCa, prostate cancer; RIP, regulated intramembrane proteolysis; sJag, soluble Jagged1 ectodomain; TEM, transendothelial migration.

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might promote disease metastasis. Furthermore, the role played by RIP-generated Jagged1 fragments in PCa has never been investigated.

Prostate cancer has a predilection to metastasize to the bone marrow stroma (BMS) and tumour-derived Jagged1 has previously been linked to the bone metastasis of breast cancer [9]. In the current study, we show that BMS can drive the expression of endogenous Jagged1 in the bone metastatic prostate cancer cell line PC3, an event which is mechanistically linked to their enhanced migration. Furthermore, the over-expression of full-length Jagged1 in PC3 cells promoted cell migration and invasion along with Notch signaling but did not promote cell proliferation. In contrast, sJag had no effect on migration, proliferation or Notch signaling but did inhibit invasion. Finally, the JICD also had no effect on migration but impaired invasion and Notch signaling whilst stimulating cell proliferation. Notably, the JICD nuclear localisation sequence was not required for any of these effects. Thus, whilst the BMS-induced expression of full-length Jagged1 can promote PC3 cell migration, RIP-generated Jagged1 fragments can have very different effects on cell behavior and Notch signaling. Therefore, the expression of the full-length ligand and production of its RIP fragments must be considered in tandem when attempting to regulate Jagged1 as a possible PCa therapy.

2. Materials and methods

2.1. Materials

The human full-length Jagged1 plasmid pIRESHyg-FL-Jag and the pIRESHyg-sJag, pIRESneo-JICD and pIRESneo-JICDΔnls plasmids were synthesized and sequenced by Epoch Biolabs (Missouri City, U.S.A.). The anti-Jagged1 N-terminal (NT) polyclonal antibody was from R&D Systems Europe Ltd. (Abingdon, U.K.) and the anti-Jagged1 C-terminal (CT) polyclonal antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, U.S.A.). The anti-actin monoclonal antibody was from Sigma–Aldrich Company Ltd. (Gillingham, U.K.). GAPDH antibody was from Abcam (Cambridge, U.K.). All other materials, unless otherwise stated, were purchased from Sigma–Aldrich Company Ltd. (Gillingham, U.K.)

2.2. Cell culture

PC3 cells were purchased from the European Collection of Cell Cultures (ECACC) and used within six months of identity verification by the Cancer Research U.K. Manchester Institute tissue typing service. All cell culture reagents were purchased from Lonza Ltd. (Basel, Switzerland); cells were cultured in Ham's F12 medium supplemented with 7% (v/v) FBS, 2 mM L-glutamine, penicillin (50 U ml^{-1}) and streptomycin (50 mg ml^{-1}) and maintained at 37 °C in 5% CO_2 in air.

Human bone marrow stroma (BMS) was obtained from volunteers undergoing surgery for benign disease and cultured as described previously [10]. Briefly, cells were grown at a density of 2×10^6 ml^{-1} in long-term bone marrow culture medium (LTBCM; Iscove's modified Dulbecco's medium at 350 mOsm, 10% (v/v) FBS, 10% (v/v) horse serum and 0.5 μM hydrocortisone) at 33 °C in 5% CO_2 in air for 4–5 weeks until haematopoietically active areas were evident.

Bone marrow endothelial cells (BMECs) used in the invasion assays were a gift from Dr. Gracia Almeida-Porada (University of Nevada, Reno NV, U.S.A.). Cell stocks were cultured in LTBCM conditioned on human bone marrow stroma in tissue culture flasks pre-coated with 50 mg ml^{-1} fibronectin in phosphate-buffered saline (PBS; 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.15M NaCl, pH 7.4). Cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air and used up to passage 20.

2.3. Generation of PC3 cell stable transfectants

Lentiviral vectors expressing full-length Jagged1 and its fragments were constructed as described previously [11,12]. Briefly, coding DNA sequences were cloned into the PRRLsin backbone and transfected into HEK293T cells along with pMDLg/pRRE, rsv-REV and pMDG.2 plasmids. Lentiviral constructs were harvested on days 2–4, pooled and stored at −80 °C. Sub-confluent PC3 cells were transduced with viral supernatant containing DEAE-Dextran (10 μg ml^{-1}) for 4 h prior to refreshing with PC3 medium. At day 5, transduced cells were FACS sorted based on green fluorescent protein (GFP) expression and expanded as stable cell lines.

2.4. Wound closure migration assays

PC3 cells were grown to confluence, the monolayer was wounded with a pipette tip and the medium removed and replaced with fresh medium containing 10 μg ml^{-1} mitomycin C. The wound was measured immediately after wounding and again at 72 h in order to permit determination of percent wound closure. In the experiments using medium conditioned on BMS (Fig. 1) the wound closure was monitored over a 17 h period as closure progressed more quickly under these conditions.

2.5. Cell viability assay

Cells were incubated with CellTiter 96® AQueous One Cell Proliferation Assay (methanethiosulfonate; MTS) solution (Promega, Wisconsin, U.S.A.) for 2 h at 37 °C. Absorbance readings at 490 nm were then taken using a Victor^2 1420 microplate reader (Perkin Elmer, Waltham, U.S.A.).

2.6. Invasion assays

PC3 cell invasion assays were performed using FlouroBlok cell culture inserts (8 μm) coated with Matrigel™ (BD Biosciences, Oxford, U.K.) and placed in a 24-well plate containing 1 ml of Dulbecco's modified Eagle's medium (DMEM)/0.1% (w/v) bovine serum albumin (BSA). In some instances an endothelial barrier was formed by culturing BMECs to confluence on top of the Matrigel™ as described previously [13]. Inserts were then transferred to new plates containing growth medium and either tissue culture plastic (TCP) alone or human BMS. PC3 cells stably expressing the various Jagged1 constructs and stained with DIL were seeded into the inserts on the surface of the Matrigel™/BMEC barrier. The invasion of cells through the barrier was assessed at hourly intervals or at an end point of 24 h by bottom reading of fluorescence on a BMG Labtech FLUOstar OPTIMA plate reader at 544/590 nm (excitation/emission filter). This plate reader is a self-contained incubator which maintains temperature and CO_2 levels at 37 °C and 5%, respectively.

2.7. Small interfering RNA transfection

Small interfering RNA (siRNA) was purchased from Thermo Scientific Dharmacon (St Leon-Rot, Germany). Cells (30–50% confluence) were treated with siRNA (25 nM) which had been pre-complexed with Dharmafect 2 reagent (St Leon-Rot, Germany) for 72 h.

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