BBA - Molecular Cell Research 1865 (2018) 25–33

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Cathepsin D non-proteolytically induces proliferation and migration in human omental microvascular endothelial cells via activation of the ERK1/2 and PI3K/AKT pathways

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ARTICLE INFO

Keywords:
Cathepsin D
Non-proteolytic
Proliferation
Migration
Angiogenesis

ABSTRACT

Epithelial ovarian cancer (EOC) frequently metastasises to the omentum, a process that requires pro-angiogenic activation of human omental microvascular endothelial cells (HOMECs) by tumour-secreted factors. We have previously shown that ovarian cancer cells secrete a range of factors that induce pro-angiogenic responses e.g. migration, in HOMECs including the lysosomal protease cathepsin D (CathD). However, the cellular mechanism by which CathD induces these cellular responses is not understood. The aim of this study was to further examine the pro-angiogenic effects of CathD in HOMECs i.e. proliferation and migration, to investigate whether these effects are dependent on CathD catalytic activity and to delineate the intracellular signalling kinases activated by CathD. We report, for the first time, that CathD significantly increases HOMEC proliferation and migration via a non-proteolytic mechanism resulting in activation of ERK1/2 and AKT. These data suggest that EOC cancer secreted CathD acts as an extracellular ligand and may play an important pro-angiogenic, and thus pro-metastatic, role by activating the omental microvasculature during EOC metastasis to the omentum.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal of all gynecological cancers. Annually, approximately 200,000 women are diagnosed with this malignancy worldwide, resulting in 125,000 disease related deaths each year [1]. Initial symptoms are often vague, frequently leading to advanced disease with widespread metastases at diagnosis which is therapeutically challenging [2]. As a result, 5 year survival is still only approximately 45% [3].

EOC primarily metastasises via the transcoelomic route, with cancer cells disseminating within the peritoneum to form secondary foci. The omentum is a common initial point of spread, with omental invasion facilitating more widespread metastasis [4]. In order to establish a secondary tumour within the omentum ovarian cancer cells must attach to the mesothelium, invade the omental tissue and then initiate angiogenesis i.e. sprouting of new blood vessels from the pre-existing vascular bed, to sustain secondary tumour growth. This process requires a complex interplay between tumour and resident cells, with secretion of growth factors and chemokines that ultimately leads to activation of a pro-angiogenic phenotype in the omental microvascular endothelial cells (ECs) and subsequently neovascularisation [5–9].

Vascular endothelial growth factor A (VEGFA) is known to be a major pro-angiogenic stimulator in many tumour types and both VEGFA expression and secretion are known to be upregulated in EOC [10,11]. However, our previous studies have indicated that angiogenic changes in the omental ECs during metastasis of ovarian cancer to the omentum are primarily driven by alternative pro-angiogenic factors secreted by EOC cells [12]. These data are supported by the observation that anti-VEGFA therapy (bevacizumab) has shown limited efficacy in patients with ovarian cancer [13], highlighting the need for a clearer understanding of the pro-angiogenic pathways involved.

One of the alternative pro-angiogenic proteins we previously identified is cathepsin D (CathD), an aspartic endopeptidase, typically involved in degrading unfolded, dysfunctional self- or foreign- proteins in lysosomes and phagosomes [14]. Although CathD is a lysosomal enzyme and its enzyme activity is usually regulated within the acidic compartment of lysosomes, it has also been reported to be secreted via, an as yet, unknown mechanism. Physiologically, CathD has been found in human, bovine and rat milk and serum [15–18], and pathophysiologically, overexpression and hypersecretion of CathD has been

Abbreviations: EOC, epithelial ovarian cancer; CathD, cathepsin D; EC, endothelial cell; ERK1/2, extracellular signal regulated kinase; AKT, protein kinase B; CAM, chorioallontoic membrane model; ECM, extracellular matrix

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http://dx.doi.org/10.1016/j.bbamcr.2017.10.005
Received 14 August 2017; Received in revised form 4 October 2017; Accepted 8 October 2017
Available online 10 October 2017
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Table 1
Concentrations of treatments added to cell proliferation assay.

Treatments	Purpose	Concentration(s)	Source
VEGF	Positive control	20 ng/ml	Peprotech (London, UK)
CathD	Treatment	20, 50, 80 ng/ml	Sigma-Aldrich (Poole, UK)
pepA	CathD inhibitor	0.1, 1, 2.5, 5 and 10 μmol/l	Sigma-Aldrich (Poole, UK)
U0126	MEK/ERK1/2 inhibitor	10 μmol/l	Stratech (Suffolk, UK)
PD 98059	MEK/ERK1/2 inhibitor	25 μmol/l	Stratech (Suffolk, UK)
LY294002	PI3K inhibitor	25 μmol/l	Stratech (Suffolk, UK)
MK2206	AKT inhibitor	5 μmol/l	Stratech (Suffolk, UK)

demonstrated in a variety of cancers including ovarian, breast, endometrial, lung and prostate, as well as malignant glioma and melanoma [12,19–32]. Indeed, we have detected CathD in the secretome of EOC cells in vitro [12], in ascites of patients suffering from ovarian cancer (unpublished data) and have shown that there is a significantly higher expression of CathD in omentum of patients with metastasised serous ovarian carcinoma compared with omentum from patients with benign ovarian cystadenoma [33].

There is increasing evidence that extracellular CathD may play a role in tumour angiogenesis including in metastasis of ovarian cancer to the omentum. In the wider cancer field CathD increases tumour vascularity in tumour xenografts in mice [18] and enhances angiogenesis in a chick chorioallontoic membrane model (CAM) [34], induces pro-angiogenic changes in more than one type of EC [12,34], as well as inducing degradation of extracellular matrix (ECM) components and release of growth factors such as basic fibroblast growth factor (bFGF) [22], and proliferation of cancer cells [35–39] and fibroblasts [40]. In relation to omental metastasis exogenous CathD induces pro-angiogenic cellular effects on disease relevant human omental microvascular ECs (HOMECs) e.g. enhanced migration structures [12] and as mentioned above is overexpressed in the omentum of patients with metastasised EOC [33].

Interestingly, although lysosomal CathD has proteolytic functions, secreted CathD has been reported to be active in both a proteolytic and non-proteolytic manner. For instance, both wild-type and mutated (ASN 231, proteolytically inactive) CathD stimulate proliferation of 3Y1-Ad12 rat tumour cells in athymic nude mice [36]. Additionally, CathD can act as a protein ligand to induce proliferation of fibroblasts [40] and MCF7 breast cancer cells [22,41]. A pro-angiogenic role for non-proteolytically active CathD has been demonstrated in studies using pepstatin A (pepA), a specific inhibitor of CathD-proteolytic activity, which inhibited CathD-induced migration and tube formation both in cultured human umbilical vein ECs (HUVECs) and angiogenesis in a CAM model [34].

Despite the emerging role for CathD in tumour growth, and the data described above suggesting specific involvement of extracellular CathD in angiogenesis in secondary omental lesions of EOC, the downstream cell signalling pathways activated by the protein are still poorly understood and remain to be elucidated. Given the therapeutic challenges posed by EOC a fuller understanding of the processes involved in secondary tumour formation may aid development of treatment strategies. We have previously published a technique for isolating disease relevant HOMECs [42] and in this study we used this cell model to investigate (a) whether CathD exerts its migratory and/or proliferative effects through a proteolytic or non-proteolytic mechanism and (b) the downstream signalling cascades activated by CathD. We demonstrate, for the first time that CathD significantly increases HOMEC proliferation and migration via proteolytic-independent mechanisms. Importantly, we also show that exogenous CathD activates the intracellular kinases ERK1/2 and AKT (S473) as part of a signalling cascade that ultimately induces a proliferative and migratory phenotype in HOMECs.

2. Materials and methods

2.1. Primary cell culture

Non-malignant omental tissue samples were collected from patients undergoing surgery at the Royal Devon and Exeter NHS Foundation Trust (Exeter, United Kingdom) with ethical approval and informed written consent. HOMECs were isolated, characterised and cultured as primary cells as previously described [42]. Briefly, HOMECs were cultured in endothelial cell growth media (MV2, PromoCell, Heidelberg, Germany) supplemented with supplied growth factors, 5% (v/v) foetal calf serum (FCS) and 0.1% (v/v) gentamycin (Sigma, Poole, UK). Cells were maintained at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

2.2. Cell proliferation assay

HOMEC proliferation was assessed using the WST-1 water soluble tetrazolium salt-1 assay (Roche, Welwyn Garden City, UK). Cells were seeded at a density of 1×10^4 cells per well in 2% (w/v) gelatin (Sigma, Poole, UK) coated 96-well plates (Greiner Bio One, Stonehouse, UK) and treated overnight in growth factor-deprived media containing 2% (v/v) FCS. After 24 h, treatments (CathD, VEGF as positive control ± inhibitors) were added at the given concentrations (Table 1) and incubated for 72 h. Subsequently, WST-1 reagent was added in a 1:10 dilution to the assay medium for 2 h incubation and absorbance was measured at 450 nm against the blank in PHERAstar BMG plate-reader. Based on the data obtained 50 ng/ml CathD was determined to be optimal for proliferation and this concentration was used for all subsequent experiments unless otherwise noted.

2.3. pH experiments

2.3.1. Measurement of pH of cell culture media during cell culture

HOMECs were seeded at a density of 3×10^5 cells per well in 6 well plates, based on preliminary optimisation experiments. After overnight incubation in growth factor depleted media as above, fresh media supplemented ± CathD (50 ng/ml) was added. Culture media was collected and pH was measured after 24, 48 and 72 h using an ABL80 FLEX blood-gas analyser (Radiometer, Crawley, UK). pH of medium-only was also measured at the beginning of incubation period.

2.3.2. Measurement of enzymatic activity of CathD at different pHs

CathD proteolytic activity was measured using a CathD-specific fluorogenic substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp) -D-Arg-NH2, (100 nmol/l, Enzo Life Sciences, Exeter, UK,), in the presence or absence of pepA (1 μmol/l) in black opaque 96-well plates (Greiner Bio One, Stonehouse, UK). Prepared buffer solutions at specific pHs containing substrate ± pepA were added to the wells (100 μl). Subsequently, 20 μl of 300 ng/ml CathD was added as required to make up the final volume of 120 μl. Control wells contained substrate or substrate and pepA, and 20 μl of corresponding pH buffer solution. The plate was shaken for 60 s in a plate-reader immediately prior to fluorescence reading at Ex/Em: 320/393. The experiment was performed

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