Cancer Letters 452 (2019) 152–157

Contents lists available at ScienceDirect

Cancer Letters

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

Mini-review

Endothelin-converting enzyme-1 in cancer aggressiveness

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ARTICLE INFO

Keywords:
ECE-1
Phosphorylation
Stability
Malignant progression
Prognosis

ABSTRACT

The endothelin-1 (ET-1) axis contributes to the pathophysiology of several cancers by promoting tumor development and progression. This peptide is activated from its precursor, big ET-1, by endothelin-converting enzyme-1 (ECE-1). Active ET-1 binds to its cognate G-coupled receptor, ET_AR, which transduces the signal to the inside of the cell. ET-1 has a short half-life of about 90 s, so its biological effects are completely dependent on its enzymatic activation by ECE-1. Expression of ECE-1 is elevated in several tumors and cancer cell lines. There are four ECE-1 isoforms —ECE-1a, -1b, -1c, and -1d— which vary in terms of their subcellular localization and, in some cases, their effects on cancer-related properties such as proliferation and invasiveness. In this article, we review findings on the role of ECE-1, particularly isoform ECE-1c, in oncogenesis and malignant progression. We also review evidence regarding ECE-1 expression in several types of tumors and cancer cell lines. Recent findings from our laboratory and others allow us to speculate on the mechanism by which ECE-1c promotes cancer aggressiveness. Finally, we evaluate potential post-translational modifications of ECE-1c, highlighting phosphorylation by several kinases, as well as evidence pointing to a putative, non-canonical, ET-1-independent mechanism for promoting invasiveness. Taken together, current evidence suggests that ECE-1c contributes to cancer aggressiveness and plays a putative role as a key regulator of cancer progression. Therefore, we propose that this protein is a promising target for prognostic and therapeutic purposes.

1. Introduction

Endothelin-1 (ET-1) expression begins with the *EDN1* gene, which encodes the 212-residue protein prepro-ET-1. This precursor is then processed by endopeptidases to produce the 39-residue protein big ET-1. Finally, active ET-1 production occurs when endothelin-converting enzyme (ECE) catalyzes the cut between Trp-21 and Val-22, generating the 21-residue peptide ET-1 [1]. Active ET-1 has a very short half-life of about 90 s, while secreted big ET1 has a relatively longer half-life of 30 min [2]. ET-1 is inactivated by the metalloprotease neprilysin [3].

ET-1 was initially described as a vasoconstrictor. However, in the last decade, the peptide has been also reported to act as a mitogen. The biological activity of ET-1 is mainly mediated by its two cognate G protein-coupled receptors, ET_AR and ET_BR, which trigger several signaling pathways related to processes such as proliferation, invasion, angiogenesis, thus suggesting a close link between ET-1 axis abnormalities and cancer progression [4,5]. Various ET-1 axis components, such as ET_AR, have been proposed as potential prognostic markers in lung, breast, colon, and other cancers. These components have been evaluated in clinical studies, but results have been modest [1]. Thus, both the physiological and pathological effects of the peptide are completely dependent on continuous processing of big ET-1 to ET-1 by the ECE [3]. In consequence, an obvious limiting step in the mitogenic role of ET-1 is the expression and therefore the activity of ECE.

We summarize here the main structural and functional differences of ECEs; the evidence regarding ECE-1 expression in several types of tumors and cancer cell lines; the role of the ECE-1c isoform in various hallmarks of cancer, with emphasis on proliferation and invasiveness; and we will discuss the potential underlying mechanisms leading to ECE-1c-mediated invasiveness. We will also provide evidence for the importance of ECE-1c phosphorylation in cancer progression, allowing propose it as a novel biomarker of poor prognosis and its potential as a target for cancer therapy.

2. Endothelin-converting enzymes

ECEs are membrane-bound metalloproteases from the M13 zinc-dependent family. These long, approximately 126-kDa polypeptides have a large, 680-residue extracellular C-terminal domain containing the catalytic site, which is post-translationally modified by N-

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https://doi.org/10.1016/j.canlet.2019.03.033
Received 25 October 2018; Received in revised form 20 March 2019; Accepted 20 March 2019

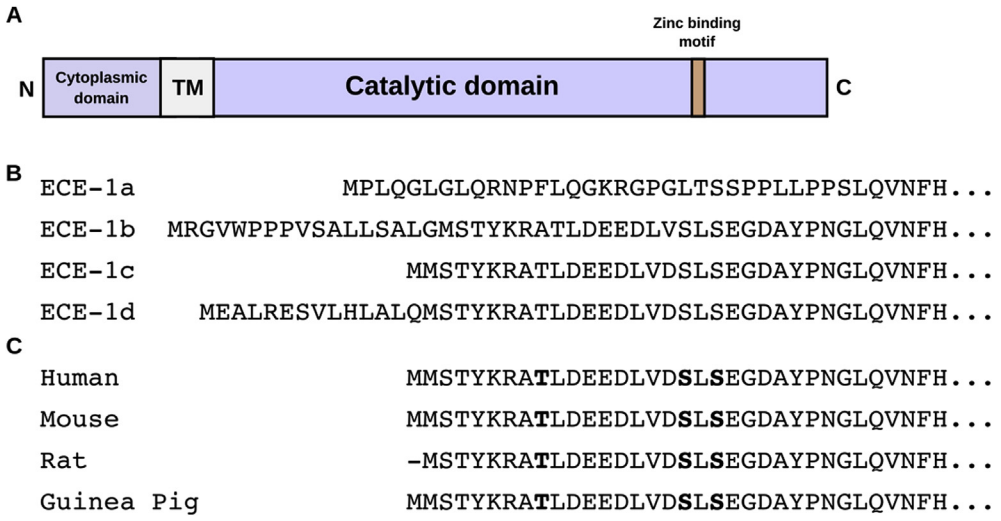

Fig. 1. Structural features of N-terminal end of ECE-1. A. Schematic view of ECE-1 and its functional domains. TM: transmembrane region. **B.** Alignment of the first 34 residues of the cytoplasmic N-terminus of ECE-1c with the other ECE-1 isoforms. **C.** Conservation of CK2 phosphorylation sites at the N-terminus of ECE-1c isoform among various animal species.

glycosylation. The C-terminal domain is followed by a single α-helix transmembrane domain containing 21 residues [6].

Three variants of ECE have been described: ECE-1, ECE-2, and ECE-3 [7–9]. The most important variants are ECE-1 and ECE-2, which process big ET-1 and other big endothelins, and to a lesser extent, ET-2 and ET-3. ECE-1 is ubiquitously expressed in most normal human tissues [10,11]. In addition to its canonical role in processing ET-1, ECE-1 regulates the traffic of G-coupled receptors of peptides unrelated to endothelins, mediating re-sensitization and activation [12–16].

Active ECE-1 is a homodimer formed by a sulfide bridge between Cys-428 of each subunit. The ECE-1 monomer is also active, but much less efficient in cleaving big ET-1 [6,17,18]. One interesting structural and functional feature of ECE-1 is a very short N-terminal cytoplasmic domain of variable length (Fig. 1), giving origin to four distinct isoforms known as ECE-1a, ECE-1b, ECE-1c, and ECE-1d [8,9,19]. All isoforms are encoded by a single 68-kb gene located on chromosome 1 and containing 19 exons. Exons 4–19 are common to all isoforms, while exons 1–3 undergo splicing to generate the four isoforms. Each isoform also has a different promoter, located upstream of the start transcription site at the first exon [11,20,21].

The subcellular location of ECE-1 is different for each isoform: ECE-1a is located within the plasma and nuclear membranes; ECE-1b is cytoplasmic, particularly at the trans-Golgi level; ECE-1c is located mainly within the plasma membrane, but also the cytoplasm; ECE-1d is located within the plasma membrane, endosomal vesicles and Golgi [9,20,22]. Homodimer formation is thought to occur for each isoform but has been also reported between ECE-1a and other isoforms [23]. All isoforms catalyze ET-1 activation with similar efficiency, with an optimum pH of 6.8, and all are inhibited by phosphoramidon [7–9]. Nevertheless, ECE-1c seems to be the most commonly-expressed isoform in all tissues [24,25].

It has been proposed that the subcellular localization of ECE-1c may be regulated by phosphorylation of its N-terminus by protein kinase PKC, increasing its localization and activity at the plasma membrane [26–29]. The N-terminus is extraordinarily conserved among many animal species, with complete identity in some residues. This finding may be relevant to the function of ECE-1c (Fig. 1). In fact, MacLeod and colleagues demonstrated that the intracellular N-terminal domain of ECE-1 has putative phosphorylation sites that are constitutively phosphorylated *in vitro* by CK1 in CHO-K1 cell lines. However, no functional relationship was established with location or activity, for example, depending on this posttranslational modification [30]. No other types of phosphorylation have been studied comprehensively in this context. Recently, Niechi and colleagues reported that phosphorylation of ECE-1c by the kinase CK2 resulted in increased protein stability as well as acquisition of malignant capabilities in colorectal cancer cells [31]. This finding suggests a putative role for this specific isoform in promoting cancer aggressiveness and thereby a poorer prognosis for patients with this disease, as we will discuss further.

Table 1
Elevated ECE-1 mRNA and protein levels identified in tumor samples, as well as modulated in cancer cell lines.

Cancer type	Hallmark studied by ECE-1 modulation/detection	References
astrocytoma	protein detected by IHC with anti-ECE-1 in tumor samples	[45]
breast	invasion by pharmacological inhibition with RO 67–7447 in MCF-7 cells protein detected by IHC with anti-ECE-1 in tumor samples	[34]
colorectal	migration & invasion by siRNA silencing and ECE-1c overexpression in DLD-1 cells	[31]
endometrial	protein detected by IHC with anti-ECE-1 in tumor samples	[41]
esophagus	protein detected by IHC with anti-ECE-1 in tumor samples	[46]
gastric	DNA polymorphisms by PCR-RFLP in tumor samples	[42]
glioblastoma	protein detected by IHC with anti-ECE-1 in tumor samples proliferation by pharmacological inhibition with RO 67–7447 in LN cells	[32]
head & neck	protein detected by IHC with anti-ECE-1 in tumor samples	[43]
lung	ECE-1 mRNA by RT-PCR and protein by ELISA/IHC in SCLC and NSCLC cells	[47]
neuroendocrine	protein detected by IHC with anti-ECE-1 in tumor samples	[10]
oral	proliferation by pharmacological inhibition and siRNA silencing in SCC cells	[33]
ovary	adhesion & invasion by siRNA silencing in ES2 and OVCAR3 cells	[35]
prostate	migration/invasion by shRNA/siRNA silencing and ECE-1c overexpression in PC3 cells	[36,37,48]
thyroid	mRNA by RT-qPCR in tumor samples	[44]

3. Role of ECE-1 in hallmarks of cancer

ECE-1 is mainly expressed in normal human tissues, but increased ECE-1 levels have been found in samples from patients with various cancers (Table 1). In addition, elevated ECE-1 mRNA and/or protein levels have been identified in cancer cell lines of different origins. In these lines, ECE-1 protein/activity levels were modulated through cDNA overexpression, specific inhibitors or siRNAs, in order to evaluate the effects of the enzyme on essentially two hallmarks of cancer, such as proliferation and invasiveness (Table 1). Interesting findings have emerged from glioblastoma LN lines treated with synthetic inhibitors, including decreased DNA synthesis and therefore inhibited proliferation [32]. Similar results were observed in oral squamous cell carcinoma cells where both a specific inhibitor and siRNA led to reduced proliferation [33]. However, cell cycle regulation or expression of proliferation/death genes were unexplored in these reports. In MCF-7 breast cancer cells, ECE-1 was inhibited with a selective inhibitor, RO 67–7447, leading to significantly decreased invasiveness [34]. Likewise, a siRNA was found to decrease invasiveness in ES2 and OVCAR3 ovarian carcinoma cells, in parallel with an improved adhesion capacity mediated by elevated E-cadherin as well as reduced N-cadherin levels and p44/42 MAPK phosphorylation [35]. A shRNA-delivery viral vector was used to silence ECE-1 in stromal or epithelial cells, significantly reducing invasion and migration of PC3 prostate cancer cells [36,37]. Interestingly, elevated ECE-1 mRNA levels were observed in a group of 44 colorectal cancer cell lines (ArrayExpress access E-MTAB-2706) derived from RNA-sequencing of 675 experimental lines [38]. In this group, DLD-1 cells showed 30% lower ECE-1 expression than HT-29 cells, which normally display low and high aggressiveness, respectively [39,40]. As expected, a siRNA against ECE-1 expression significantly decreased migration and invasiveness of DLD-1 colorectal cancer cells [31].

Conversely, ECE-1 overexpression also increases invasiveness in cancer cell lines. Notably, Lambert and coworkers showed that ECE-1c overexpression or silencing in PC3 prostate cancer cells could increase or reduce invasiveness, respectively. These authors also showed that the ECE-1a isoform could play the opposite role, as its overexpression decreased invasiveness in PC3 cells. The ECE-1b and ECE-1d isoforms appeared to have no effect on invasiveness [36,48]. Similar findings were reported for ECE-1c overexpression in DLD-1 colorectal cancer cells, which resulted in significantly enhanced migration and invasiveness [31]. The specific role of ECE-1c and its relative isoforms in hallmarks of cancer other than invasiveness has not been assess yet, leaving important questions unanswered.

One unresolved point is the mechanism underlying ECE-1c overexpression in cancer cells. At the transcriptional level, a simple *in silico* analysis of the ECE-1c promoter predicts the existence of a canonical Wnt response element (WRE) in position −455, inside an important regulatory sequence. However, pharmacological activation and inhibition of this pathway were assessed in colon cancer and non-tumor cell lines, which had a null effect on regulation of ECE-1c expression (Tapia J.C., personal communication). In addition, Whyteside and coworkers showed that ECE-1c expression in PC3 prostate cancer cells is regulated by alternative polyadenylation, through production of truncated 3′-UTR transcripts, leading to increased protein synthesis [37,49]. While this mechanism may explain the reported invasiveness of PC3 cells observed elsewhere [36,48], the authors did not experimentally address this phenomenon, which therefore remains to be demonstrated. However, given that the only difference between ECE-1 isoforms is a small sequence at the N-terminal cytoplasmic end, ECE-1c-mediated invasiveness likely depends on a covalent modification within this region.

4. Mechanisms leading to ECE-1c-mediated invasiveness

How ECE-1c promotes invasiveness in cancer cells is another unanswered question. Perhaps the most obvious mechanism is increased ET-1 production and ET_AR activation. In fact, silencing ECE-1 was shown to decrease ET-1 production, as well as MMP-2 activity and invasiveness, in OVCAR3 and ES2 ovarian cancer cells. These effects were reverted by ET-1 supplementation, suggesting that ECE-1 may exert its role through continuous production of ET-1 [35]. Nevertheless, in PC3 prostate cancer cells, ECE-1c modulated invasiveness, but ET-1 supplementation only marginally rescued the effect of ECE-1c silencing [48]. This finding suggests an apparently ET-1-independent mechanism for ECE-1c-mediated invasiveness. Interestingly, this mechanism also seems to function in other cancer models. In DLD-1 and HT-29 colorectal cancer cells, the ECE-1 inhibitor SM19712 exerted a similar decrease in invasiveness as compared to a siRNA against ECE-1c [31].

An alternative mechanism accounting for ET-1-independent ECE-1c-promoted invasiveness could be related to the activity of focal adhesion kinase (FAK), a known enzyme that regulates both migration and invasion in various cells. In PC3 prostate cancer cells overexpressing ECE-1c, siRNA-mediated ECE-1 silencing or ECE-1 overexpression led to a significant decrease or increase in FAK phosphorylation, respectively, although the report did not address whether this FAK-related activity correlated with invasiveness [37]. Alternatively, it is also well known that FAK is an upstream regulator of the PI3K/AKT signaling pathway and *vice versa* in various cancer cell lines [50]. Indeed, Akt has been identified as a downstream target of ET-1 through ET_AR signaling in prostate [51] and ovarian [52] cancer cells. Therefore, ECE-1c-mediated increases in invasiveness could be attributable to activation of Akt and then FAK, although the underlying mechanisms would remain unknown.

Another mechanism that might complement the process described above and further explain ECE-1c-promoted invasiveness is covalent modification of the N-terminus. ECE-1c activity, location, and sorting may be regulated by phosphorylation at the N-terminus by protein kinases MAPK and PKC in non-tumor cells [27,28], as well as by CK2 in colon cancer cells [31]. This kinase is elevated in a wide variety of tumors, and abnormally high levels are associated with increased growth and proliferation [53]. Therefore, CK2 has been proposed as a marker of poor prognosis in patients with acute myeloid leukemia, lung, pancreatic, and prostate cancers, among others [54–57]. In colorectal cancer, CK2 has been identified as a metastasis-associated gene in a proteomic study using different cell lines [58]. Furthermore, CK2 levels are correlated with poor prognosis [59]. At the cellular level, CK2 promotes survival of colorectal and breast cancer cells by activating the Wnt/β-catenin signaling pathway through phosphorylation of AKT at Ser-129 [60,61], which increases the expression of many β-catenin targets, including the inhibitor of apoptosis survivin [40].

Indeed, ET-1 itself is another β-catenin target whose expression is increased in several cancers [1]. Moreover, a new transcriptional and post-translational mechanism linking CK2 and the ET-1 axis may have been unveiled in colorectal cancer (Fig. 2). In this context, CK2 was demonstrated to phosphorylate the N-terminus of ECE-1c, preventing its presumably proteasome-dependent degradation, which promoted migration and invasiveness of DLD-1 colorectal cancer cells. Additionally, CK2 inhibition led to a decrease in ECE-1c protein stability [31], which is consistent with the elevated CK2 protein and activity levels found in these and other cancer cells [53].

5. Concluding remarks/perspectives

The pathological effects of the ET-1 axis are dependent on activation by ECE-1. While it is well-known that ECE-1 mRNA and protein levels are elevated in biopsies from patients with several types of cancer, the molecular basis underlying this increased expression remains unknown. In addition, it would be interesting to study whether ECE-1c levels correlate with disease stage and/or metastatic potential. Likewise, it may also be interesting to explore the existence of mutations at the putative ECE-1c phospho-sites in patient biopsies in order to correlate this molecular event with clinical outcomes. However, there are not

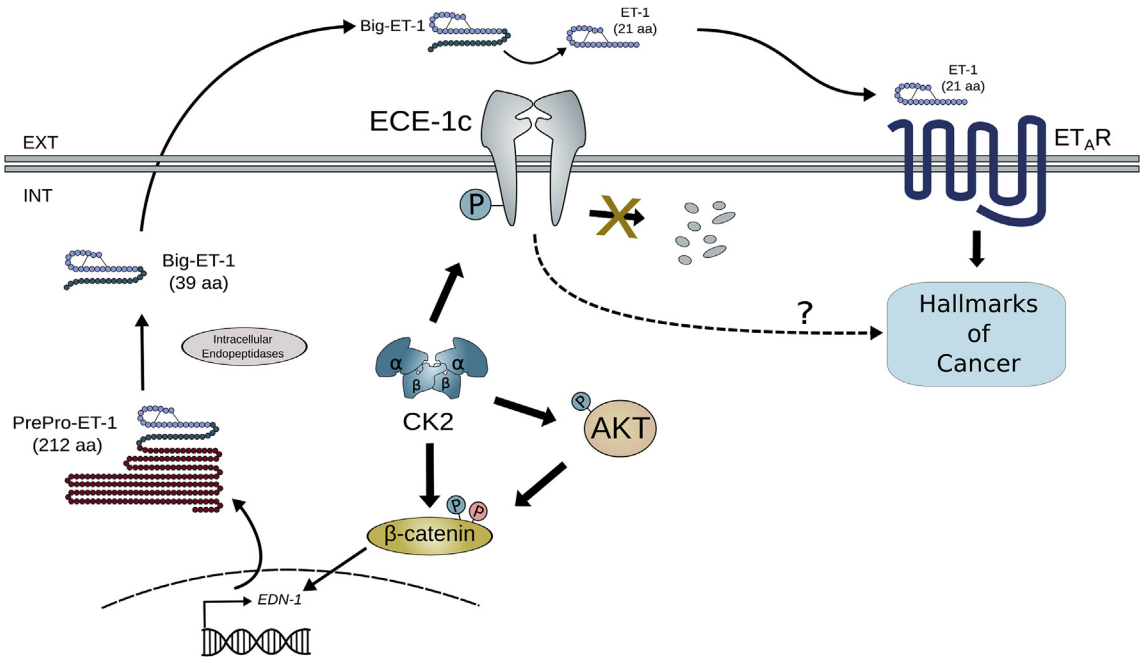

Fig. 2. A comprehensive model for transcriptional and post-translational regulation of endothelin-converting enzyme-1c and its function at the ET-1 axis. At transcriptional level, big ET-1 expression may be promoted by the Wnt/β-catenin signaling pathway, which is upregulated by phosphorylation of CK2 and Akt [40,61]. At post-translational level, ECE-1c stability is promoted by CK2-dependent phosphorylation, which presumably leads to an increased ET-1 activation. Elevated ET-1 levels can trigger several signaling pathways through binding to the cognate ET_A receptor [1], which would lead to several hallmarks of cancer [62].

genetic mutations or genomic markers that account for increased ECE-1c expression in cancer cells.

The individual contribution of the various ECE-1 isoforms to malignant progression remains an open question. Initially, increase in invasiveness of prostate cancer cells was only observed when the ECE-1c isoform was over-expressed, while ECE-1a played the opposite role, although the mechanism accounting for this effect was not explored [48]. Just seven years later, isoform ECE-1c was shown to promote invasion in colon cancer cells by a mechanism involving the specific phosphorylation of the N-terminus by the protein kinase CK2 [31]. Although the mechanism by which ECE-1c promotes aggressiveness is still unknown, the different subcellular locations of the isoforms, the ability of ECE-1a to heterodimerize with other isoforms and the different N-terminus of ECE-1a, could provide an answer. CK2 should not phosphorylate the N-terminus of ECE-1a (see Fig. 1), whose heterodimerization (if any) with phospho-ECE-1c may be inhibitory, perhaps as a consequence of a change in subcellular localization. Alternatively, ECE-1c-promoted aggressiveness may occur through an ET-1-independent or non-canonical process, for example, through FAK or MMP activation, or some combination of the two, as suggested elsewhere [36,37]. Nevertheless, as shown for example with β-arrestin in ET_AR signaling [63], for this non-canonical process there is to date no report of a factor involved in the increased stability of ECE-1c after phosphorylation by CK2, or if that hypothetical factor is responsible for the putative FAK-dependent migration.

The literature reviewed here only allows to suggest that ECE-1c may play a role as a prognostic marker, at least in colon and prostate cancers. For cancer therapy, however, decreasing ET-1 production via inhibition of ECE-1 may be an alternative to the disappointing findings obtained with ET_AR antagonists in clinical trials [64–68]. Despite the obvious disadvantages of targeting the extracellular catalytic domain of ECE-1, which is the same for all isoforms, some pharmacological inhibitors have been used *in vitro* in cancer cells of different origins, albeit not having yet been tested in clinical trials. Also, dual inhibition of ECE-1 and neprilysin has been tested but only for treating hypertension in a high-risk population [64]. Moreover, the intracellular effect of ECE-1 inhibition may not only affect extracellular production of ET-1 (see Fig. 2), as ET-1 supplementation only marginally rescues the effect of ECE-1c silencing in invasiveness of PC3 cells [48]. Therefore, blockage of the N-terminal phosphorylation of ECE-1c by CK2 may be an interesting alternative to decrease cancer aggressiveness. Although the easiest way to target this region is through a phospho-specific antibody, the unknown identity of the residues phosphorylated by CK2 precludes this possibility [31]. Another way may be designing cell penetrating peptides like biased antagonists, such as the β-arrestin biased ET_AR antagonists which have the potential to achieve better results in ovarian cancer therapy [69]. In conclusion, the incoming evidence will allow to postulate phospho-ECE-1c as a novel biomarker of poor prognosis and, perhaps in the future, as a target for therapy of patients with cancer.

Conflicts of interest

The authors disclose no potential conflicts of interest.

Funding

The study was sponsored by the Fondo Nacional de Desarrollo Cientifico y Tecnologico-Chile (FONDECYT 1160889).

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