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Carboxypeptidase E promotes cancer cell survival, but inhibits migration and invasion

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ABSTRACT

Carboxypeptidase E (CPE), a prohormone processing enzyme is highly expressed and secreted from (neuro)endocrine tumors and gliomas, and has been implicated in cancer progression by promoting tumor growth. Our study demonstrates that secreted or exogenously applied CPE promotes survival of pheochromocytoma (PC12) and hepatocellular carcinoma (MHCC97H) cells under nutrient starvation and hypoxic conditions, but had no effect on their proliferation. CPE also reduced migration and invasion of fibrosarcoma (HT1080) cells. We show that CPE treatment mediates survival of MHCC97H cells during metabolic stress by up-regulating the expression of anti-apoptotic protein BCL-2, and other pro-survival genes, via activation of the ERK1/2 pathway.

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1. Introduction

Cancer is the second leading cause of death worldwide, yet despite extensive research, the mechanisms involved in tumor growth, survival and metastatic potential are still not fully understood [1]. Neuroendocrine cancers such as pheochromocytoma, are some of the least understood forms of the disease [2]. It is well established that various types of (neuro)endocrine tumors, including pheochromocytomas, lung small cell carcinomas [3], insulinomas [4], breast adenocarcinoma [5] and glioblastomas [6] secrete large numbers of hormones and growth factors, as well as their processing enzymes, such as carboxypeptidase E (CPE) and peptidylglycine alpha-amidating mono-oxygenase [7]. Among the prohormone processing enzymes, CPE has been the most studied with respect to tumorigenesis. CPE is a multifunctional protein that in addition to its enzymatic function, sub-serves many essential non-enzymatic roles in the endocrine and nervous system, besides a role in cancer (for a review, see Ref. [8]) and it has been recently shown to act as a trophic factor [9].

High throughput microarray studies have correlated elevated CPE mRNA levels with metastasis in a number of non-endocrine cancers [10], although the form of CPE protein translated from these mRNAs was not analyzed. Recently, an N-terminally truncated splice isoform of CPE, known as CPE-ΔN was identified and shown to be a molecule that induces tumor metastasis [11]. It is elevated in highly metastatic hepatocellular carcinoma (HCC), breast, colon and head and neck cancer cell lines compared to their isogenic low metastatic counterparts. CPE-ΔN is also a powerful biomarker in diagnosing and predicting future metastasis in several cancers including HCC, pheochromocytoma/paraganglioma (PHEO/PGL) and colorectal cancer [11,12]. CPE-ΔN, up-regulates the expression of *Nedd9*, a metastatic gene in a HDAC1/2–dependent manner [11]. In addition to the CPE-ΔN splice variant just described, the primary gene product of the CPE gene, full length wild-type CPE (referred to as CPE) is normally localized in regulated secretory granules of (neuro)endocrine cells. Within these granules it processes peptide hormone and neuropeptide intermediates to their mature bioactive peptides. CPE is secreted from (neuro)endocrine tumor and glioblastoma (GBM) cell lines. It was found that CPE acts as a pro-growth, but anti-metastatic factor [6]. Another recent study has shown that CPE acts extracellularly as a negative regulator of the canonical Wnt signaling pathway [13] and as a neurotrophic factor to protect neurons against oxidative stress induced cell death [9] or during chronic stress [14]. However, the mechanism of action of CPE in these functions is not fully understood.

Neuroendocrine tumors such as PHEO/PGL express both CPE and CPE-ΔN. However, we found that only high levels of the splice isoform, but not CPE, was correlated with enhanced PHEO/PGL tumor metastasis and poor prognosis [11]. In this paper, we investigated whether secreted CPE from these tumors could serve a different role, perhaps as a survival/proliferative factor, similar to that reported for glioblastoma [6]. To determine this possibility,

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we added neutralizing CPE antibodies to the culture medium of rat pheochromocytoma-derived PC12 cells [15] and found that the cells showed significantly decreased survival in serum free media. We then investigated the mechanism of action of CPE as a survival factor, using a highly metastatic HCC cell line (MHCC97H) lacking background endogenous CPE [11], as a model. We examined the survival effect of exogenously added CPE during metabolic stress on these cells, as well as the signaling pathway and expression of downstream genes. We also used the highly invasive and aggressive fibrosarcoma cell line HT1080, which does not express wild type CPE (see Suppl. Fig. S1), but only CPE-ΔN, to assay the effects of CPE on invasion and migration.

2. Materials and methods

2.1. Cell lines

Rat pheochromocytoma PC12 cells were obtained from ATCC (Manassas, VA). The human HCC cell line, MHCC97H, was obtained from the Liver Cancer Institute, Fudan University (Shanghai, China). The HT1080 fibrosarcoma cells were obtained from TREVIGEN Inc. (Gaithersburg, MD).

2.2. Secretion studies for PC12 cells

PC12 cells were seeded on 10 cm dishes and allowed to grow to 70–85% confluency in DMEM medium (ATCC, Manassas, VA) supplemented with 10% FBS and 5% horse serum. Cells were later washed with 1× PBS and 1.5 mL of serum free DMEM medium was added to the cells. After 24 h the medium was collected and briefly centrifuged to remove debris. The supernatant containing secreted proteins was collected and stored at −80 °C for later use.

2.3. Treatment of PC12 cells with CPE antibodies and cell survival assay

PC12 cells were seeded on a 96 well plate and allowed to grow to 50–75% confluency in DMEM supplemented with 10% FBS and 5% horse serum. Cells were washed with 1X PBS and then low glucose (1 g/L D-glucose) serum free (LGSF) DMEM medium was added to induce stress. To neutralize the action of any CPE secreted from the cells, 0.25 μg of rabbit polyclonal anti-CPE IgG (Anti-CPE #6135, generated in our Lab, was custom made against an 18 aa synthetic peptide corresponding to aa #362-379 of mouse CPE, NCBI accession# NP_038522 and coupled to KLH at the N-terminus), was added to each of 6 experimental wells. Another six wells had 0.25 μg of IgGs from a non-specific rabbit antibody added to the medium to serve as an antibody control. Six wells were left completely untreated in serum free DMEM medium. This plate containing the three groups of were immediately placed inside the Modular Incubator Chamber (MIC-101) (Billups-Rothenburg), sealed, and flushed with a low oxygen mixture comprising of 1% oxygen, 5% carbon dioxide, and 94% nitrogen for 60 s at a rate of 40 L/min after which it was placed into the 37 °C incubator. The chamber was subsequently flushed every 12 h as described above, to ensure that a uniform hypoxic environment was maintained. Six wells in a separate plate were untreated in DMEM complete media and placed in the incubator for 24 h to serve as a normal cell control. After the incubation for 24 h under the various conditions all cells were harvested to evaluate the cytotoxic effects of nutrient deprivation and hypoxia on the cells using the a LDH assay (Promega, Madison, WI) according to manufacturer's instructions.

2.4. Cell viability and proliferation analysis

Cell viability or cytotoxicity was evaluated by determining the levels of LDH released into the culture media after various treatments [16]. LDH was assayed with a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit according to the manufacturer's instructions (Promega, Madison, WI). Proliferation was evaluated by quantification of the DNA binding dye, DRAQ5 that fluoresces at 680 nm upon binding to DNA and is a direct measure of cell density. HCC cells were plated at a density of 40–50% and allowed to grow to ~75% confluency overnight at 37 °C, 5% CO_2 in DMEM medium supplemented with 10 % fetal calf serum, sodium pyruvate (0.11 mg/mL), penicillin (100 U/mL) and streptomycin (100 mg/mL). The medium was then removed and after rinsing with 1× PBS, the cells were incubated with medium with or without (control) purified recombinant CPE protein (200 nM). The cells were stained with DRAQ5 (BioStatus Ltd., UK) according to the manufacturer's instructions at the following time points: 0, 8, 24, 34, 48, 56, and 72 h. Cells stained with DRAQ5 were analyzed on the Odyssey infrared imager at 680 nm. Integrated intensity values were obtained and used to generate a growth curve.

2.5. Metabolic stress paradigm for HCC cells

HCC cells were plated at a density of 40–50% and allowed to grow to ~75% confluency overnight in as mentioned above. The following day the medium was changed to LGSF medium and the plates were immediately placed inside the Modular Incubator Chamber (MIC-101) (Billups-Rothenburg), sealed, and flushed with a low oxygen mixture comprising of 1% oxygen (as described above for PC12 cells). The chamber was placed into the 37 °C incubator and subsequently flushed every 12 h as described above to ensure that a uniform hypoxic environment was maintained.

2.6. Treatment of HCC cells with exogenous CPE

HCC cells were treated with different concentrations (50–400 nM) of recombinant mouse full length CPE (custom expressed in HEK293 cells and purified by Creative Biolabs, Shirley, NY) by adding it to the cell medium at zero time point of each experiment. A concentration of 200 nM was established as the most effective dose for eliciting an increase in the phosphorylation of ERK 1/2. Since the CPE is very stable in the media, no additional CPE was added throughout the experiment. It was also found that human CPE lacking the C-terminal cytoplasmic tail (purchased from Sino Biological Inc., Beijing, China) gave the same results as full length mCPE. For inhibition of CPE activity, the HCC cells were treated with 5 μM guanidino-ethyl-mercapto-succinic acid (GEMSA), a competitive inhibitor of CPE with a Ki ~8 nM [17] along with 200 nM CPE. For inhibition of ERK 1/2, cells were treated with 10 μM U0126 for 30 mins with/without 200 nM CPE. After 30 min the cells were harvested and proteins were analyzed by Western blotting.

2.7. Western blot for CPE, BCL-2, ERK 1/2, GSK3β/α, AKT, β-catenin and PKC

Proteins from cell lines were prepared using cell lysis buffer (Cell Signaling Technology) supplemented with Complete Inhibitor Cocktail (Roche) to prevent protein degradation. The cell lysate was collected and centrifuged at 15,000*g* for 10 min at 4 °C and the protein concentration in the supernatant determined using the Bio-Rad Protein Assay. Twenty μg of protein was denatured at 95 °C for 3 min, ran on 4–12% SDS–PAGE gels, and then transferred onto nitrocellulose membrane (Invitrogen), according to standard protocols. After blocking with 5% nonfat milk at 4 °C for