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Alternate AChE-R variants facilitate cellular metabolic activity and resistance to genotoxic stress through enolase and RACK1 interactions

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ABSTRACT

Tumorogenic transformation is a multifaceted cellular process involving combinatorial protein–protein interactions that modulate different cellular functions. Here, we report apparent involvement in two independent tumorogenic processes by distinct partner protein interactions of the stress-induced acetylcholinesterase AChE-R and N-AChE-R variants. Human testicular tumors showed elevated levels of N-terminally extended N-AChE-R compared with healthy tissue, indicating alternate promoter usage in the transformed cells. Two-hybrid screens demonstrate that the C-terminus common to both N-AChE-R and AChE-R interacts either with the glycolytic enzyme enolase or with the scaffold protein RACK1. *In vitro*, the AChE-R C-terminal peptide ARP elevated enolase's activity by 12%, suggesting physiological relevance for this interaction. Correspondingly, CHO cells expressing either human AChE-R or N-AChE-R but not AChE-S showed a 25% increase in cellular ATP levels, indicating metabolic significance for this upregulation of enolase activity. ATP levels could be reduced by AChE-targeted siRNA in CHO cells expressing AChE-R but not AChE-S, attributing this elevation to the AChE-R C-terminus. Additionally, transfected CHO cells expressing AChE-R but not N-AChE-R showed resistance to up to 60 μM of the common chemotherapeutic agent, *cis*-platinum, indicating AChE-R involvement in another molecular pathway. *cis*-Platinum elevates the expression of the apoptosis-regulator p53-like protein, p73, which is inactivated by interaction with the scaffold protein RACK1. In co-transfected cells, AChE-R competed with endogenous RACK1 for p73 interaction. Moreover, AChE-R-transfected CHO cells presented higher levels than control cells of the pro-apoptotic TAp73 as well as the anti-apoptotic dominant negative ΔNp73 protein, leading to an overall decrease in the proportion of pro-apoptotic p73. Together, these findings are compatible with the hypothesis that in cancer cells, both AChE-R and N-AChE-R elevate cellular ATP levels and that AChE-R modifies p73 gene expression by facilitating two independent cellular pathways, thus conferring both a selective metabolic advantage and a genotoxic resistance.

1. Introduction

Accentuated cell metabolism and loss of cell cycle/apoptotic control are key characteristics of the cancer cell [1,2]. Much effort has been invested in delineating the molecular mechanisms underlying these processes and finding ways to control them, but the complete

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picture is far from being fully understood. Changes in tumor cell physiology involve numerous proteins and signaling pathways [1], but many more proteins may be involved. One example is the stress-induced isoform of acetylcholinesterase, AChE-R. AChE levels are elevated in a wide variety of tumors [3] and expression levels were correlated with malignancy in ovarian cancer [4]. In particular, AChE-R is notably elevated in various tumor types [5], including lymphocytic [6] and glial tumor cancer cells [7]. AChE-R levels in abnormal cells are directly correlated with cell proliferation, both in CHO cells [8] and in hyper-proliferating thymocytes [6]. Additionally, the identified elevated AChE-R can be one of two different variants. Transcription initiation from two alternative sites in the ACHE promoter produces transcripts encoding for "classic" or "extended" N-terminal sequence of the AChE protein isoforms (termed N-AChE, Fig. 1A) [9]. At their 3′ end, AChE mRNA transcripts undergo alternative splicing, yielding catalytically active protein isoforms differing in their C-terminus. The synaptic isoform (AChE-S, also called AChE-T [10]) is translated from transcripts containing exon 6, whereas the transcripts containing pseudointron 4 encode the AChE-R isoform. By performing yeast two-hybrid screens with AChE or the AChE-R C-terminus as bait, we and others have previously identified proteins that bind AChE or AChE-R [11–14]. These partner proteins modulate the AChE-related molecular pathways and AChE-R's non-enzymatic functions in both nerve cells [15] and blood cells [16]. In differentiating sperm cells, we found that AChE-R interacts with the glycolytic enzyme enolase and the scaffold protein RACK1 [14]. This raised the question about the functional role and outcome of such interactions in tumor cells.

Enolase-α is a ubiquitously expressed glycolytic enzyme, which catalyses the conversion of 2-phosphglyceric acid to phosphoenol pyruvate. Enolase-α levels are notably increased under hypoxic stress, presumably to enhance glycolytic rate to compensate for the decreased aerobic respiration [17,18]. The metabolism of cancerous cells is often characterized by greater dependence on glycolysis [19]. Sperm cells, which are also greatly dependent on glycolysis, display elevated motility and ATP levels in AChE-R transgenic mice compared with those of controls [14]. This raised the possibility that AChE-R could also have an effect on tumor cell metabolism through its interaction with enolase.

RACK1 is a propeller-shaped protein with multiple protein-binding domains. In testis of transgenic mice over-expressing AChE-R, the cell types expressing RACK1 also displayed a higher fraction of apoptotic cells as compared with strain-matched controls. As a major junction of molecular processes in the cell, RACK1 has also been implicated in regulating the cell cycle and apoptosis [20,21].

One of the apoptosis-related proteins that interact with RACK1 is p73 [21], a transcription factor homologous to p53. p73 transcripts undergo alternative splicing at their 3′ end, yielding several different isoforms with alternative C-termini. The alpha isoform, which binds RACK1 through its unique C terminal region, is the longest form of the protein with no exons spliced out. Transcription of the p73 gene initiates from two alternative promoters, yielding two p73 isoforms that differ in their N-terminal sequences. The full length protein is transcriptionally active (TAp73), whereas the truncated protein lacks the transactivation domain (ΔNp73). p73 expression has been implicated in several cancers [22,23]. As a result of regulation of promoter selection, p73α can therefore be either pro- or anti-oncogenic, depending on whether anti- or pro-apoptotic isoforms (ΔNp73 or TAp73 respectively) are expressed [23]. Indeed, shift in the ratio of the pro-apoptotic TAp73 to the anti-apoptotic ΔNp73 correlates with sensitivity of the tumor cells to chemotherapy [24].

To understand AChE-R's role in cancer cells, we examined the effect of AChE-R–enolase interaction on cellular metabolism in transformed testicular tissue. Additionally, we tested if AChE-R–RACK1 interaction competes with p73–RACK1 interaction and assessed possible consequences to the sensitivity of cultured CHO cells to the chemotherapeutic drug, *cis*-platinum.

2. Materials and methods

2.1. Tissue samples

Human testicular biopsies containing normal tissue obtained during removal of a testicular tumor were fixed in formalin and embedded in paraffin. Histological examination performed by the pathology department in the Sourasky Medical Center identified the tumors as carcinoma *in situ* or seminoma. We received tissue slices containing both tumor tissue and adjacent normal seminiferous tubules.

2.2. Immunohistochemistry

Immunohistochemistry was performed as described elsewhere [14]. Briefly, antigen retrieval was performed in Citrate buffer pH6; polyclonal antibodies directed to the C-terminal peptide of AChE-R [25] and at the common region (N19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:100 v/v, and antibodies directed at the N-terminal domain [9] were diluted 1:50. Biotinylated secondary antibodies were detected with strepavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Fast Red (Roche Molecular Biochemicals, Mannheim, Germany) was used as a chromogenic substrate. Immunostaining was viewed with a Zeiss Axioplan microscope (Zeiss, Göttingen, Germany) and captured with Real-14^{TM} digital color camera (CRi, Boston, MA, USA). Stained tissue area was measured by the ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA).

2.3. Enolase assay

Activity of enolase purified from rabbit muscle (Sigma, St. Louis, MO, USA) was determined by reaction conditions described elsewhere [14]. Synthetic peptides constituting the 26-amino-acid-long C-terminal sequence of human AChE-R (ARP26) or 23 C-terminal residues of AChE-S (ASP23) [26] were also diluted in enolase reaction buffer. Peptide sequences were as follows. ARP26: GMQGPAGSGWEEGSGSPPGVTPLFSP; ASP23: WSSYMVH-