Biochemical and Biophysical Research Communications 430 (2013) 1283–1288

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Acidosis blocks CCAAT/enhancer-binding protein homologous protein (CHOP)- and c-Jun-mediated induction of p53-upregulated mediator of apoptosis (PUMA) during amino acid starvation

Christopher B. Ryder [a], Karen McColl [b], Clark W. Distelhorst [a,b,*]

[a] Department of Pharmacology, Case Western Reserve University School of Medicine, Case Comprehensive Cancer Center, and University Hospitals Case Medical Center, Cleveland, OH 44106, USA

[b] Department of Medicine, Case Western Reserve University School of Medicine, Case Comprehensive Cancer Center, and University Hospitals Case Medical Center, Cleveland, OH 44106, USA

ARTICLE INFO

Article history:
Received 21 November 2012
Available online 19 December 2012

Keywords:
Acidosis
Amino acids
Apoptosis
PUMA
CHOP
c-Jun

ABSTRACT

Cancer cells must avoid succumbing to a variety of noxious conditions within their surroundings. Acidosis is one such prominent feature of the tumor microenvironment that surprisingly promotes tumor survival and progression. We recently reported that acidosis prevents apoptosis of starved or stressed lymphoma cells through regulation of several Bcl-2 family members (Ryder et al., JBC, 2012). Mechanistic studies in that work focused on the acid-mediated upregulation of anti-apoptotic Bcl-2 and Bcl-xL, while additionally showing inhibition of glutamine starvation-induced expression of pro-apoptotic PUMA by acidosis. Herein we report that amino acid (AA) starvation elevates PUMA, an effect that is blocked by extracellular acidity. Knockdown studies confirm that PUMA induction during AA starvation requires expression of both CHOP and c-Jun. Interestingly, acidosis strongly attenuates AA starvation-mediated c-Jun expression, which correlates with PUMA repression. As c-Jun exerts a tumor suppressive function in this and other contexts, its inhibition by acidosis has broader implications for survival of cancer cells in the acidic tumor milieu.

1. Introduction

Tumor growth and progression breeds an increasingly inhospitable local environment, thereby imposing numerous obstacles to further expansion. In order to survive, cancer cells must overcome microenvironmental stresses such as hypoxia, nutrient limitation, and acidic stress [1]. Adaptations that facilitate malignant progression in the face of these harsh cell-extrinsic conditions are critical to the oncogenic process. In fact, deregulated responses to external stimuli figure prominently among the hallmarks of the disease [2].

One characteristic perturbation within the tumor micro-environment is the development of extracellular acidosis. Whereas most normal tissues exhibit a $pH_e \sim 7.4$, numerous studies find intratumoral pH_e measurements between 6.5 and 7.0 [3]. Despite this extracellular acidity, cancer cells maintain a slightly alkaline intracellular space. This pH gradient reversal is critical, as intracellular acidosis can activate nucleases and the apoptotic cascade [4,5]. Indeed, extracellular acidosis is toxic to some cell types [6,7]. In stark contrast, numerous reports show that acidosis promotes therapeutic resistance and invasive phenotypes [8–10]. Mechanisms continue to be elucidated for this surprising tumorigenic role for acidosis.

We recently reported that acidosis inhibits apoptosis of starved or stressed lymphoma cells through regulation of multiple members of the Bcl-2 family [11]. This group consists of over 20 proteins that share homology in at least one of four distinct Bcl-2 homology (BH) domains [12]. These proteins primarily control entry into the intrinsic apoptosis cascade, with some members promoting cell death and others having an inhibitory role. Our work revealed that induction of anti-apoptotic family members Bcl-2 and Bcl-xL by acidosis contributes significantly to its cytoprotective effect and that the elevation of these pro-survival proteins requires GPR65, an acid-sensing G protein-coupled receptor (GPCR). Additionally, we found acidification to strongly block starvation-induced elevation of pro-apoptotic PUMA (p53-upregulated mediator of apoptosis) at both the mRNA and protein level. Yet the mechanism

Abbreviations: AAR, amino acid response; Apaf-1, apoptotic protease-activating factor-1; ATF, activating transcription factor; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein; Bim, Bcl-2-interacting mediator of cell death; CHOP, CCAAT/enhancer-binding protein homologous protein; eIF2α, eukaryotic initiation factor-2alpha; GCN2, general control nonderepressed 2; GPCR, G protein-coupled receptor; Mdm2, Murine double minute 2; PARP, poly-(ADP-ribose) polymerase; pH_e, extracellular pH; PUMA, p53-upregulated mediator of apoptosis.

* Corresponding author. Address: Charles S. Britton II Hematology/Oncology, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106, USA. Fax: +1 216 368 1189.

E-mail address: cwd@case.edu (C.W. Distelhorst).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.11.136

for repression of PUMA by acidosis remains undetermined. Further inquiries in this direction stand to uncover pH-dependent regulatory factors that contribute to acidosis-mediated evasion of apoptosis.

Though originally discovered to be induced by p53, PUMA expression has since been shown to be controlled by numerous factors, primarily at the level of transcription (reviewed in [13]). Activation of this BH3-only protein is known to occur in response to diverse stimuli such as DNA damage, ER stress and growth factor withdrawal. Involved transcription factors include CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) and c-Jun, among others. PUMA exerts its pro-apoptotic function by directly activating Bax and Bak, leading to mitochondrial outer membrane permeabilization and apoptosis [14]. It is no surprise then that expression levels of this tumor suppressive gene are decreased in several tumor types, though genetic inactivation does not seem to be contributory [13]. Further understanding of PUMA repression in cancer remains an important area of investigation.

In this study we show that PUMA upregulation during amino acid (AA) starvation requires induction of both CHOP and c-Jun. Interestingly, we find that acidosis strongly represses starvation-induced c-Jun levels while not affecting CHOP expression. We propose that CHOP and c-Jun cooperate to elevate pro-apoptotic PUMA and that acidosis represses PUMA elevation by blocking c-Jun expression. These findings highlight a novel mechanism for the promotion of cancer cell survival mediated by tumor-associated acidity.

2. Materials and methods

2.1. Cell culture

Maintenance and experimental conditions for wild type and Bcl-2-expressing WEHI7.2 cells were previously described [11]. Initial cell density of $3–6 \times 10^5$ cells/mL was used for all experiments. Control and acidic pH media were set to 7.55 ± 0.1 and 6.50 ± 0.1, respectively.

2.2. Immunoblot analysis

Protocol was described previously [11]. Antibodies used include: anti-PUMA, anti-Bim, anti-CHOP, anti-c-Jun, anti-cleaved caspase-3, and anti-PARP from Cell Signaling, and anti-Actin from Sigma. Protein expression was visualized with ECL reagent or ECL Prime (GE Healthcare).

2.3. RNA isolation and RT-PCR

RNA isolation and RT-PCR were performed as described previously [11]. All assays were created from Roche universal probe library. Primers are as follows ($5' \rightarrow 3'$): CHOP (Forward (F)-gcgacagagccagaataaca, Reverse (R)-gatgcacttccttctggaaca); c-Jun (F-ccagaagatggtgtggtgttt, R-ctgaccctctccccttgc); JunB (F-ccacggaggg-gagagaaaatc, R-agttggcagctgtgcgtaa); c-Fos (F-gggacagcctttcctac-tacc, R-agatctgcgcaaaagtcctg).

2.4. RNA interference

For gene knockdown, 10^7 cells were electroporated with ON-TARGETplus SMARTpool siRNA (Dharmacon, Lafayette, CO), as described previously [11]. Cells were transiently transfected with either non-targeting control, CHOP-specific or c-Jun-specific siRNA.

2.5. Statistical analysis

All statistical analyses show the highest level of significance for repeated measures ANOVA with Tukey–Kramer post-test. Analyses were performed using GraphPad Prism software. Densitometric analysis was done using ImageJ software. Error bars represent ± standard error of the mean for at least three experiments.

3. Results

3.1. Starvation of different AAs causes similar acid-inhibitable increases in apoptosis and PUMA levels

The goal of this study was to understand the mechanistic basis for the pH-dependent induction of PUMA during glutamine starvation of lymphoma cells. As a first step to address this question, we needed to understand the nature of the apoptotic stress. This knowledge would then inform our later investigation into the mediating factors. As glutamine is a vital fuel source for cancer cells in addition to its roles as a precursor for protein synthesis and transamination reactions, we investigated whether starvation of different AAs, namely the two sulfur-containing amino acids cysteine and methionine, would elicit a similar response and whether this cell death would also be inhibited by acidosis. Importantly, sulfur-containing AAs are among those decreased in the tumor microenvironment [15]. Therefore, we set up a direct comparison of glutamine versus cysteine/methionine starvation of WEHI7.2 murine lymphoma cells in the presence or absence of extracellular acidity. We found similar levels of cell death upon starvation of either AA(s) after 12 h (Fig. 1A). Furthermore, acidosis inhibited the cell death in either starvation condition. In CEM-C7 human lymphoma cells, AA starvation caused minimal cell death before 72 h (data not shown). As expected from our previous work we found the cell death to be apoptotic, as starvation markedly increased cleavage of caspase-3 and PARP (Fig. 1C). Acidosis strongly attenuated the appearance of these apoptotic markers. In contrast, the appearance of cell death upon glucose starvation only became detectable at 24 h, when glutamine starved cells are nearly all dead (Fig. 1B and [11]) These data suggest that the apoptosis is an AA withdrawal response rather than a metabolic starvation.

We next tested whether the two AA starvation protocols regulate the pro-apoptotic Bcl-2 family members PUMA and Bim similarly, as shown for glutamine withdrawal in our earlier studies [11]. In fact, robust elevation of both proteins occurred after starvation of either AA(s). In line with our previous findings acidosis strongly blocked PUMA induction, whereas the degree of Bim protein repression by acidity varied between experiments (Fig. 1C). Since acidosis elevates PUMA-interacting proteins Bcl-2 and Bcl-xL, we next examined the regulation of PUMA upon AA starvation of Bcl-2-overexpressing WEHI7.2 cells. This experiment tested whether the increase in anti-apoptotic Bcl-2 proteins by acidosis mediates changes in pro-apoptotic family members. AA starvation and acidosis had similar effects on PUMA levels in Bcl-2-expressing compared to wild type cells (Fig. 1C and D), suggesting that the control of PUMA levels occurs independently of expression changes for its inhibitory binding partners.

3.2. Starvation-induced CHOP mediates PUMA and Bim elevation

Because PUMA and Bim upregulation occurred in response to an AA starvation insult, we next focused on factors induced or activated by AA starvation that could, in turn, mediate increases in these BH3-only proteins. Among the downstream components of the AA response (AAR) is CHOP (C/EBPζ, CHOP10, DDIT3, GADD153) [16]. Importantly, CHOP has been shown to mediate