EXPERIMENTAL CELL RESEARCH ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Available online at www.sciencedirect.com

ScienceDirect

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

Research Article

BCL-2 family protein, BAD is down-regulated in breast cancer and inhibits cell invasion

Maria Cekanova[a,*,1], *Romaine I. Fernando*[b,1,2], *Nalin Siriwardhana*[c], *Mugdha Sukhthankar*[d,3], *Columba de la Parra*[e], *Jirayus Woraratphoka*[b,4], *Christine Malone*[f], *Anders Ström*[g], *Seung J. Baek*[d], *Paul A. Wade*[f], *Arnold M. Saxton*[c], *Robert M. Donnell*[d], *Richard G. Pestell*[h], *Suranganie Dharmawardhane*[e], *Jay Wimalasena*[b,**]

[a]*Department of Small Animal Clinical Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN, USA*
[b]*Department of Obstetrics and Gynecology, Graduate School of Medicine, Medical Center, The University of Tennessee, Knoxville, TN, USA*
[c]*Department of Animal Science, The University of Tennessee, Knoxville, TN, USA*
[d]*Department of Biomedical and Diagnostics Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN, USA*
[e]*Department of Biochemistry, School of Medicine, University of Puerto Rico, Medical Sciences Campus, San Juan, PR, USA*
[f]*Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA*
[g]*Center for Nuclear Receptors and Cell Signaling, Department of Biology and Biochemistry, University of Houston, Houston, TX, USA*
[h]*Department of Cancer Biology, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA*

ARTICLE INFORMATION

Article Chronology:
Received 31 October 2012
Received in revised form
27 October 2014
Accepted 24 November 2014

ABSTRACT

We have previously demonstrated that the anti-apoptotic protein BAD is expressed in normal human breast tissue and shown that BAD inhibits expression of cyclin D1 to delay cell-cycle progression in breast cancer cells. Herein, expression of proteins in breast tissues was studied by immunohistochemistry and results were analyzed statistically to obtain semi-quantitative data. Biochemical and functional changes in BAD-overexpressing MCF7 breast cancer cells were evaluated using PCR, reporter assays, western blotting, ELISA and extracellular matrix invasion

Abbreviations: AIF, apoptosis inducible factor; AP-1, activator protein-1; AKT, protein kinase B; Apaf-1, apoptosis protease activating factor-1; BAD, Bcl-2-associated death promoter; BCL-2, B-cell lymphoma 2; BCLxL, B-cell lymphoma-extra large; BH3, Bcl-2 homology domain 3; CXCL12/SDF1, stromal cell-derived factor-1; CXCR4, chemokine receptor type 4; DM, double mutant; ECL, enhanced chemiluminescence; EGFP, enhanced GFP; EMSA, electrophoretic mobility shift assay; EMT, epithelial-mesenchymal transition; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERK, extracellular signal-regulated kinases; FADD, Fas-associated protein with death domain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GSK3β, glycogen synthase kinase 3 beta; HRP, horseradish peroxidase; IHC, immunohistochemistry; p, phospho; Ras/MEK/ERK, MAPK signaling pathway; JNK, c-Jun kinase; MCL1, myeloid leukemia cell differentiation protein-1; MMP10, metalloproteinase-10; MTA3, metastasis-associated protein-3; Sp1, specificity protein-1; STAT, Signal transducer and activator of transcription; TMA, tissue microarrays; TIMP2, metallopeptidase inhibitor 2; TRE, transcription response elements; VEGF, Vascular endothelial growth factor

*Corresponding author.
**Correspondence to: The University of Peradeniya, Medical Faculty, Department of Physiology, Peradeniya, Sri Lanka.
E-mail addresses: mcekanov@utk.edu (M. Cekanova), jwimalas@gmail.com (J. Wimalasena).
[1] These authors equally contributed to this work.
[2] Laboratory of Tumor Immunology and Biology, Center for Cancer Research, NCI, NIH, Bethesda, MD, USA.
[3] Biomedical Biotechnology Research Unit, Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown, South Africa.
[4] Suranaree University of Techonology, Nakhon Ratchasima, Thailand.

http://dx.doi.org/10.1016/j.yexcr.2014.11.016

Keywords:
BAD
Extracellular matrix invasion
Breast cancer
Metastasis

assays. Compared to normal tissues, Grade II breast cancers expressed low total/phosphorylated forms of BAD in both cytoplasmic and nuclear compartments. BAD overexpression decreased the expression of β-catenin, Sp1, and phosphorylation of STATs. BAD inhibited Ras/MEK/ERK and JNK signaling pathways, without affecting the p38 signaling pathway. Expression of the metastasis-related proteins, MMP10, VEGF, SNAIL, CXCR4, E-cadherin and TIMP2 was regulated by BAD with concomitant inhibition of extracellular matrix invasion. Inhibition of BAD by siRNA increased invasion and Akt/p-Akt levels. Clinical data and the results herein suggest that in addition to the effect on apoptosis, BAD conveys anti-metastatic effects and is a valuable prognostic marker in breast cancer.

Introduction

Most apoptosis regulators, including BCL-2 family members, are typically localized to the intracellular membranes, cytoplasm, or mitochondria [1–4]. We previously demonstrated that the BCL-2 antagonist, BAD is localized to the nucleus, in addition to the cytoplasm in normal human breast tissue and that BAD prevents cyclin D1 transcription, hence cell cycle progression in breast cancer cells [5]. Al-Bazz et al. [6] and we [7] reported that BAD is localized to both the nucleus and cytoplasm in breast cancer tissue, suggesting a nuclear role for BAD. Other BH3 proteins, BIM and BCL-2 are also localized to the nucleus [1–3,8] suggesting that BCL-2 family proteins may have nuclear roles [9]. Our observations suggest that although BCL-2 and BAD have opposing effects on apoptosis in vitro [10,11], their cell-cycle-related functions could be comparable (see *Discussion*).

Many clinical studies suggest that BCL-2 expression is a strong predictor of overall and disease-free survival in breast cancer patients. BCL-2 is a favorable and superior prognostic marker [12,13] independent of lymph node status, tumor size, grade, and other biomarkers including estrogen receptor α (ERα) [14]. This is in marked contrast to the majority of in vitro studies, where BCL-2 is depicted as a pro-survival or cancer-promoting factor [10,11]; however, BCL-2 has a variety of non-apoptotic functions in vitro [10,11,15–20] as does another BCL-2 family protein MCL1 [16,18,21]. BID has been demonstrated to have a role in inflammation and immunity independent of apoptosis [22]. In recent studies non-apoptotic roles of BAD were shown to include: blood glucose regulation, cooperation with p53 in the mitochondria, cell-cycle regulation, and pro-survival functions [23–28]. Many of the proteins that have critical roles in apoptosis also have non-apoptotic functions, including cytochrome C, which is a key player in the intrinsic apoptosis pathway and is required for oxidative phosphorylation-linked electron transport. In addition to their well-established roles in apoptosis, functions for caspases have been described in cell-cycle entry, cell maturation, immune system function [29,30], differentiation [31], and other apoptosis-unrelated functions [32,33]. Other pro-apoptotic molecules, e.g. apoptosis inducing factor (AIF), Endo G and Omi [34,35] also have pro-survival effects [36,37].

As a continuation of our previous work on the role of BAD in breast cancer cells [5,38], we evaluated the importance of BAD in breast cancer progression both in vitro and patient samples. We demonstrated that BAD regulated several key molecules governing epithelial–mesenchymal transition (EMT), which thereby modulated the extra-cellular matrix invasion of breast cancer cells in vitro. This is the first demonstration of anti-invasive effects of a BCL-2 family protein on breast cancer cells.

Materials and methods

Cell Lines and plasmid vectors

MCF7 human breast cancer cell line, and conditional and transient overexpression BAD constructs have been previously described [5,39,40].

Antibodies

Following antibodies were used in this study: BAD (H-168), phospho-AKT (S473), phospho-ERK1, ERK1, ERβ, Actin, Flag, phospho-c-Jun, c-Jun, phospho-JNK, JNK, phospho-p38, CXCR4, β-catenin, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-BAD (S136), Sp1 (Upstate, Lake Placid, NY); AKT (Cell Signaling, Danvers, MA); cyclin D1 (Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK); SNAIL (Abcam, Cambridge, MA); GFP (Sigma, St. Louis, MO) and MTA3 antibody was described in [41]. Secondary antibodies: Amersham (San Diego, California) and Chemicon (Millipore, Billerica, MA).

Immunohistochemistry

Formalin-fixed and paraffin-embedded blocks of human normal ($n=3$) and neoplastic breast tissue ($n=4$) were purchased from SeraCare GCI Global Repository (West Bridgewater, MA) and breast tissue microarrays (TMAs) BR801, BR722 were purchased from US Biomax, Inc. (Rockville, MD). Slides were incubated overnight in a humid chamber at 4 °C with appropriate concentrations of primary antibodies. Incubation with diluent alone served as negative controls. Slides were washed and incubated with biotinylated Super Sensitive Link followed by HRP-conjugated Super Sensitive Label (BioGenex, Fremont, CA). Diaminobenzidine (Vector, Burlingame, CA) was used as the substrate. Nuclei were lightly counterstained with hematoxylin (Richard Allen Scientific, MI). Stained slides were reviewed by a certified pathologist (RLD) and the staining intensities were scored on a 0–3 scale (0=no staining, 1=mild staining, 2=moderate staining, and 3=marked staining). The scores of the staining were averaged for statistical analyses.

Western blot

Protein samples were separated on 10% SDS-PAGE and transferred to PVDF or nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies, HRP-labeled secondary antibodies, and followed by ECL to detect proteins [5].

Please cite this article as: M. Cekanova, et al., BCL-2 family protein, BAD is down-regulated in breast cancer and inhibits cell invasion, Exp Cell Res (2014), http://dx.doi.org/10.1016/j.yexcr.2014.11.016