Differential Expression of Antiapoptotic Gene BAG-1 in Human Breast Normal and Cancer Cell Lines and Tissues

Xiaolong Yang, Yawei Hao, Zhihu Ding, Alan Pater, and Shou-Ching Tang

Basic Medical Sciences, Faculty of Medicine, Memorial University of Newfoundland [X. Y., Y. H., Z. D., A. P.], and Newfoundland Cancer Treatment and Research Foundation, Dr. H. Bliss Murphy Cancer Centre [S-C. T.], St. John’s, Newfoundland, Canada A1B 3V6

ABSTRACT

BAG-1 is an antiapoptotic protein that binds to and enhances the antiapoptotic activity of Bcl-2. It binds several growth factor and hormone receptors and modulates their function. BAG-1 was also shown recently to be expressed as four protein isoforms, p50, p46, p33, and p29, through alternative translation initiation. Although many apoptosis-associated genes have been linked to oncogenesis of human breast cancer, the role of BAG-1 has not been fully elucidated. In this study, we examined the expression of BAG-1 RNA or protein isoforms and its interacting antiapoptotic proteins, Bcl-2 and Bcl-XL, in breast normal and tumor cell lines and tissues by Northern or Western blot analysis. We provide convincing evidence that both BAG-1 RNA and protein are overexpressed in human breast cancer cell lines. More importantly, we found that the expression of two isoforms of BAG-1, p46 and p33, was also much higher in breast primary tumors. The expression of Bcl-2 and Bcl-XL correlated with that of BAG-1 in breast normal and carcinoma cell lines but not tissues. Our study suggests that BAG-1 isoforms may serve as a molecular marker, independent of Bcl-2 and Bcl-XL, for human breast cancer.

INTRODUCTION

Programmed cell death, or apoptosis, is a ubiquitous process in which cells commit suicide under certain environmental conditions. Increasing evidence suggests that apoptosis is controlled through the expression of many cellular proteins, including inducers (Bax, Bcl-Xs, Bad, Bak, and Bik) and inhibitors (Bcl-2, BAG-1, Bcl-XL, and Mcl-1) of apoptosis (1–7). It has been suggested that defects in apoptosis-regulating genes are important for pathogenesis of human cancers (8–10). This hypothesis is supported by the observation that p53, an inducer of apoptosis, is frequently deleted or mutated in various human cancers, whereas Bcl-2, an inhibitor of apoptosis, is overexpressed in lymphomas and in breast, lung, prostate, cervical, colorectal, and other cancers (11–18).

BAG-1 is a recently discovered antiapoptotic protein capable of interacting with and enhancing the activity of Bcl-2 (6). Furthermore, it inhibits apoptosis induced by apoptotic stimuli or cytokine withdrawal in mouse NIH3T3 cells and lymphocytes (6, 19, 20). Recently, BAG-1 was also shown to bind the plasma membrane-associated tyrosine kinase growth factor receptors (hepatocyte growth factor and platelet-derived growth factor receptors) and enhance the ability of these receptors to inhibit apoptosis (21). In addition, BAG-1 was shown to bind several hormone receptors, such as ER, androgen receptor, and glucocorticoid receptor, and modulate their function (22–24). BAG-1 therefore acts as a multifunctional antiapoptotic protein capable of interacting with various cellular proteins.

Although the molecular function of human BAG-1 has been extensively studied, the role of BAG-1 in the carcinogenesis of human cells is uncertain. Recently, enhanced expression of BAG-1 in breast cancer tissues was found by Zapata et al. (25). However, only one normal breast and three breast carcinoma tissues were used for Western blot in their study. In addition, we have recently shown that BAG-1 is expressed as four protein isoforms, p50, p46, p33, and p29, through alternative translation initiation (26). However, the role of different BAG-1 isoforms in tumorigenesis has not been elucidated. In this study, we show that BAG-1 isoforms can be differentially expressed in cell lines in vitro compared with tissues in vivo. Furthermore, we provide evidence that the BAG-1 isoforms p46 and p33 are overexpressed in both breast carcinoma cell lines and tissues and may play an important role, independent of Bcl-2 and Bcl-XL, in breast carcinogenesis.

MATERIALS AND METHODS

Cell Culture and Breast Biopsies. Three normal human mammary gland cell lines (HS574, HS578, and HS787) and nine human breast carcinoma cell lines (BT-20, BT-474, HS578T, MCF-7, SK-BR-3, MDA-MB-157, MDA-MB-231, MDA-MB-436, and MDA-MB-468) were used in this study. All of the cell lines were obtained from American Type Culture Collection (Manassas, VA). Except for SK-BR-3, which was cultured in McCoy’s 5a medium, all of the cell lines were maintained in DMEM containing 10% heat-inactivated FCS.

The breast normal and carcinoma tissues were obtained from the General Hospital, the Health Science Center (St. John’s, Newfoundland, Canada) and National Cancer Institute

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2 To whom requests for reprints should be addressed, at 300 Prince Philip Drive, St. John’s, Newfoundland, Canada A1B 3V6. Phone: (709) 737-4227; Fax: (709) 737-6795; E-mail: stang@nctrf.nf.ca.

3 The abbreviations used are: ER, estrogen receptor; ECL, enhanced chemiluminescence; mAb, monoclonal antibody.
of Canada Manitoba Breast Cancer Tumor Bank (Winnipeg, Manitoba, Canada). Tissue samples were excised fresh from operative specimens. Each specimen was either cut into 1-mm segments or sliced into 50-μm-thick pieces and stored at −270°C before protein extraction. Sections were histologically classified with respect to the presence of breast normal and invasive carcinoma tissues. Care was taken to exclude all normal tissue from invasive tumor samples using histological examination.

RNA and Protein Extractions from Human Breast Cell Lines or Biopsies. Total RNA was extracted from breast normal and carcinoma cells grown to 70% confluence using the CsCl gradient centrifugation method (27). Proteins were extracted from 10^7 cultured cells or 0.1-g biopsies by lysis in 1 ml of ice-cold extraction buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 50 μg/ml aprotinin] for 30 min and centrifuged at 12,000 g for 10 min.

Northern and Western blot analysis. Northern and Western blot analysis of cellular gene expression was performed as described previously (28), with minor modifications. In brief, for Northern blot, 20 μg of total RNA were separated in denaturing 1% agarose gels and transferred to Hybond nitrocellulose membranes (Amersham Corp., Arlington Heights, IL). The blots were hybridized with [32P]-labeled human BAG-1 cDNA probe in Rapid-hyb hybridization buffer (Amersham Corp.) at 65°C for 1 h, washed twice with 2× SSC/0.1% SDS at room temperature for 15 min and twice with 0.1× SSC/0.1% SDS at 65°C for 10 min, and then exposed to Kodak BioMax film overnight. Actin was used as an internal control.

For Western blot analysis, 10 μg of protein were fractionated by 10% SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Corp.) under semidry conditions. Immunodetection was done using the ECL system (Amersham Corp.), according to the manufacturer’s instructions. Actin was used as an internal control. The level of each protein was determined by densitometry and normalized by comparing its level with that of actin. Anti-BAG-1 mAb was generated as described previously (26). Anti-Bcl-2 and anti-β-actin mAbs and anti-Bcl-X_L polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The statistical significance of the difference of protein expression between normal and tumor tissues was analyzed by the Student t test.

RESULTS

Overexpression of BAG-1 RNA in Human Breast Cancer Cell Lines. To investigate the role of BAG-1 in oncogenesis of human breast cells, we examined the expression of BAG-1 RNA in three cell lines (HS574, HS578, and HS787) derived from normal breast epithelium and in nine breast cancer cell lines (BT-20, BT-474, HS787T, MCF-7, SK-BR-3, MDA-MB157, MDA-MB231, MDA-MB436, and MDA-MB468) by Northern blotting (Fig. 1). Whereas the expression of BAG-1 RNA was low in all of the three normal breast cell lines, it was significantly higher (increased 3.75–7.76-fold) in seven of the nine breast cancer cell lines. Only two breast carcinoma cell lines, HS578T and MDA-MB157, expressed relatively low levels of BAG-1 RNA (0.70-fold and 1.78-fold, respectively, relative to BAG-1 RNA level in normal breast cell lines; Table 1).

Overexpression of Four BAG-1 Protein Isoforms in Breast Cancer Cell Lines: Correlation with the BAG-1-Interacting Proteins Bcl-2 and Bcl-X_L. Consistent with BAG-1 RNA expression, the expression of the four BAG-1 protein isoforms, p50, p46, p33, and p29 (26), was low in the
three normal breast cell lines. BAG-1 protein expression was higher relative to normal cell lines in seven of the nine breast carcinoma cell lines. Only HS578T and MDA-MB-157 expressed relatively low levels of BAG-1 protein (0.65-fold and 0.75-fold, respectively, relative to the BAG-1 protein level in normal breast cell lines, Fig. 2; Table 1). Because BAG-1 binds and synergizes with Bcl-2 to suppress apoptosis, we examined whether the expression of BAG-1-interacting antiapoptotic proteins, Bcl-2 and Bcl-XL, was also higher in breast cancer cell lines. Interestingly, Bcl-2 protein level was higher in six of the nine breast cancer cell lines, and the level of doublet Bcl-XL was higher in five of the nine cancer cell lines (Fig. 2). The expression level of each isoform of BAG-1 protein was quantified by normalizing the optical density of bands at medium exposure on X-ray film. BAG-1 protein level represents the total band intensity of p50, p46, p33, and p29 relative to that of β-actin.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Status</th>
<th>BAG-1 RNA</th>
<th>BAG-1 Protein</th>
<th>Bcl-2 protein</th>
<th>Bcl-XL protein</th>
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<tbody>
<tr>
<td>HS574</td>
<td>Normal</td>
<td>1.00</td>
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<tr>
<td>HS787</td>
<td>Normal</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>BT-20</td>
<td>Adenocarcinoma</td>
<td>3.75</td>
<td>1.75</td>
<td>2.82</td>
<td>13.00</td>
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<td>BT-474</td>
<td>Ductal carcinoma</td>
<td>6.77</td>
<td>4.80</td>
<td>98.38</td>
<td>272.78</td>
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<tr>
<td>HS578T</td>
<td>Ductal carcinoma</td>
<td>0.70</td>
<td>0.65</td>
<td>1.04</td>
<td>35.02</td>
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<tr>
<td>MCF-7</td>
<td>Adenocarcinoma</td>
<td>6.24</td>
<td>2.63</td>
<td>51.57</td>
<td>51.47</td>
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<tr>
<td>SK-BR-3</td>
<td>Adenocarcinoma</td>
<td>6.91</td>
<td>2.91</td>
<td>1.06</td>
<td>101.86</td>
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<td>MDA-MB-157</td>
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<td>1.78</td>
<td>0.75</td>
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<td>1.11</td>
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<tr>
<td>MDA-MB-231</td>
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<td>188.44</td>
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<tr>
<td>MDA-MB-436</td>
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<td>2.94</td>
<td>19.24</td>
<td>34.91</td>
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<tr>
<td>MDA-MB-468</td>
<td>Adenocarcinoma</td>
<td>7.76</td>
<td>2.94</td>
<td>16.50</td>
<td>68.27</td>
</tr>
</tbody>
</table>

Table 1 Relative expression of BAG-1, Bcl-2, and Bcl-XL in breast normal and carcinoma cell lines

Overexpression of BAG-1 Protein in Breast Carcinoma Tissues. To test the relevance of BAG-1 protein overexpression in breast carcinoma cell lines to the in vivo condition, we examined the expression of BAG-1 protein in 24 normal and 45 invasive carcinoma breast tissues. The expression level of each isoform of BAG-1 protein was quantified by normalizing the level of each band in Western blots with that of the actin control. Fig. 3 shows an example of the Western blots, and Fig. 4 shows the distribution of protein level among individual samples. In general, both the mean level and percentage of sample positivity of p46 and p33 were markedly higher in breast carcinoma than in normal tissues (Figs. 3 and 4; Table 2). The mean level and percentage of p46-positive samples are 0.0% and 4.2%, respectively, in normal tissues, whereas they are 0.12% and 80.0%, respectively, in carcinoma tissues. Similarly, the mean level and percentage of p33-positive samples are 0.01% and 4.2%, respectively, in normal tissues, whereas they are 0.19% and 62.2%,
respectively, in carcinoma tissues. Statistical analysis also shows that the expression of p46 and p33 in normal and breast cancer tissues is significantly different (P < 0.001; Table 2). Although the level of p50 in breast carcinomas was statistically higher than that in normal mammary tissues, its mean level or percentage of BAG-1-positive tissues was the same or almost the same (normal versus tumor tissue: mean, 0.99 versus 1.18, P < 0.05; % positive, 100% versus 100%; Fig. 4; Table 2). p29 was detected only in one breast carcinoma tissue (Fig. 3). Whereas p33 was the predominant isoform of BAG-1 protein in breast normal and malignant cell lines (Fig. 2), p50 was the predominant isoform in breast normal and carcinoma tissues (Fig. 3).

In contrast to our experiment using breast cell lines, Bcl-2 and Bcl-XL were not overexpressed in breast cancer tissues (Fig. 3, 5). No difference was observed in the level of Bcl-XL between breast normal and cancer tissues (Table 2). Moreover, the mean level of Bcl-2 was 10-fold lower in breast cancer than in normal tissues.

**DISCUSSION**

Although breast cancer is the leading cause of cancer-associated death for women (29), the molecular mechanism for carcinogenesis has not been fully understood. Recently, a number of observations have suggested that dysregulation of apoptosis plays an important role in the pathogenesis of human cancers (for reviews, see Refs. 8–10). Inactivation of proapoptotic proteins such as p53 and Bax, and activation of antiapoptotic proteins such as Bcl-2 and Bcl-XL, has been observed in a variety of human cancers (11–18). Although the expression of the apoptosis-associated genes, p53, bcl-2, and bax, has been extensively studied in breast cancer, conflicting results have been reported in the literature (14, 30–32). Bcl-2 was found to be overexpressed in some breast carcinoma cell lines (14), but not changed or even lower in some other breast cancer cell lines or tissues (30, 31, 33). Reduced expression of the proapoptotic gene bax was observed in breast cancer cell lines and tissues, and the expression of mRNA for the antiapoptotic genes bcl-2 and bcl-XL was similar in breast normal and cancer tissues (30, 31). Heterogeneous expression of Bax was also detected in primary breast tumors (34). To fully understand the role of apoptosis in the pathogenesis of breast cancer, it is necessary to examine the expression of genes other than p53, bax, and bcl-2 that are involved in apoptosis.

In this study, we examined the expression of BAG-1, an antiapoptotic protein, and its interacting proteins in breast normal and carcinoma cell lines using Western blot analysis. We provide convincing evidence that the expression of BAG-1 protein was increased in breast carcinoma versus normal cell lines and tissues. Although immunohistochemistry is not quantitative or able to distinguish isoforms, similar trends were observed when comparing BAG-1 staining in breast carcinoma with normal tissues using this method(4) (25). Our Western blot analysis of the four BAG-1 isoforms shows that there was a significant difference in normal versus cancer cells in vivo compared with tissues in vitro. Whereas all of the BAG-1 protein isoforms were overexpressed in breast carcinoma cell lines (Fig. 1), only p46 and p33 were higher in breast carcinoma tissues.

**Fig. 4** Normalized expression level of BAG-1 p50, p46, and p33 proteins in breast normal and carcinoma tissues. The level of each protein in Western blot was determined with a densitometer. The relative levels of p50, p46, and p33 are indicated for each tissue sample by the ratio of proteins:actin.

4 Z. Ding and X. Yang, unpublished data.
Because the levels of BAG-1 RNA and protein were closely correlated in breast cell lines, enhanced expression of BAG-1 protein isoforms in these carcinoma cell lines is likely due to increased production of BAG-1 RNA. However, all BAG-1 isoforms are translated from a single mRNA transcript (26). Therefore, a higher level of p46 and p33 but not p50 in breast carcinoma tissues may be caused by relatively increased usage of the second and third initiation codons during translation of BAG-1 in breast cancer tissues. Similarly, a significant difference was also found for Bcl-2 and Bcl-X\textsubscript{L} in breast cell lines and tissues. One possible cause for the differential expression of BAG-1, Bcl-2, and Bcl-X\textsubscript{L} in breast normal and carcinoma tissues is the changes occurring in cell growth requirements or cell selection during initial or after prolonged culture of normal or tumor cells in vitro. In addition, changes for gene expression during the menstrual cycle in tissues (35) but not cell lines may be another cause for the difference. Limited sample size could also contribute to the difference. Additional experiments using a greater number of breast cell lines and tissues are needed to confirm the above possibilities.

Finding much higher expression of p46 and p33 but not p50 in breast cancer tissues is of interest. Our previous study indicated that different BAG-1 isoforms may have distinct subcellular localization (26), suggesting that they may have distinct function in the cells. Interestingly, p50, but not p46 or p33, was recently found to enhance androgen receptor-mediated transactivating activity (23). Therefore, the four BAG-1 isoforms may have different roles in transcription regulation, apoptosis, and tumorigenesis. Recently, enhanced expression of BAG-1 p33 in three breast primary tumors was also observed by Zapata et al. (25). Because other BAG-1 isoforms, p50, p46, and p29, were not examined in their study, the differential expression of BAG-1 isoforms in breast carcinoma was not clear. The molecular mechanism for the overexpression of p46 and p33 in breast carcinoma tissues is still unknown. Additional experiments need to be performed to elucidate the role of BAG-1 isoforms in carcinogenesis of breast cells by transfecting different BAG-1 isoforms into human breast carcinoma cells and examining the tumorigenicity of BAG-1-overexpressing cells in nude mice.

A synergistic effect of BAG-1 and Bcl-2 on antiapoptosis has been shown previously by Takayama et al. (6). However, the expression level of BAG-1 and Bcl-2 was only correlated in breast cell lines but not in tissues. Whereas the expression level of BAG-1 increased in invasive breast carcinoma tissues, that of Bcl-2 decreased. Similar results were obtained by Zapata et al.

### Table 2

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Normal (N = 24)</th>
<th>Tumor (N = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>% positive</td>
</tr>
<tr>
<td>p50</td>
<td>0.99 ± 0.85</td>
<td>100.0</td>
</tr>
<tr>
<td>p46</td>
<td>0.00 ± 0.00</td>
<td>4.2</td>
</tr>
<tr>
<td>p33</td>
<td>0.01 ± 0.03</td>
<td>4.2</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>1.12 ± 0.97</td>
<td>91.7</td>
</tr>
<tr>
<td>Bcl-X\textsubscript{L}</td>
<td>0.10 ± 0.12</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The level of each protein in the Western blot was quantified by densitometry and normalized using the β-actin control. The mean ± SD of all normal or tumor samples was expressed in arbitrary units; % positive represents the percentage of samples with detectable BAG-1.

\textsuperscript{b} Difference is of low (P < 0.05) statistical significance by Student’s t-test comparison of the different protein levels in normal and tumor samples.

\textsuperscript{c} Difference is of high (P < 0.001) statistical significance by Student’s t-test comparison of the different protein levels in normal and tumor samples.

\textsuperscript{d} Difference is of no statistical significance by Student’s t-test comparison of the different protein levels in normal and tumor samples.
(25) using an immunohistochemical assay for BAG-1 and Bcl-2. In addition, although a strong correlation between Bcl-2 immunostaining and ER positivity in invasive carcinoma of the breast was reported previously (36), no such correlation was found between BAG-1 level and ER positivity. Furthermore, no correlation between BAG-1 and Bcl-X<sub>L</sub> was found. Interestingly, although the expression of BAG-1 was not correlated with conventional prognostic factors such as age, histology, and stage, its expression was associated with a shorter disease-free and overall survival in a multivariate analysis (37). Therefore, BAG-1 may serve as a molecular marker, independent of Bcl-2, Bcl-X<sub>L</sub>, and ER, for breast oncocogenesis in vivo.

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**REFERENCES**


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