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Different expression patterns of Bcl-2 family genes in breast cancer by estrogen receptor status with special reference to pro-apoptotic Bak gene

H Eguchi*[,1], K Suga[1,3], H Saji[2], M Toi[2], K Nakachi[1] and S-I Hayashi[1]

[1] Hormone Associated Cancer Research Group, Saitama Cancer Center Research Institute, 818 Komuro, Ina-machi, Kita-adachi-gun, Saitama 362-0806, Japan
[2] Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo, 113-0021, Japan
[3] Current address: Department of Blood Transfusion, Saga Medical School
* Corresponding author: H Eguchi, Hormone Associated Cancer Research Group, Saitama Cancer Center Research Institute, 818 Komuro, Ina-machi, Kita-adachi-gun, Saitama 362-0806, Japan Tel: +81 48 722 1111 (Ext.4621); Fax: +81 48 722 1739; E-mail: hide@cancer-c.pref.saitama.jp

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Abstract

Oncogenic and anti-apoptotic Bcl-2 is expressed much less in estrogen receptor α (ERα) negative breast cancers, which show more malignant phenotypes, than ERα-positive, indicating that some other Bcl-2 family member(s) are involved in the apoptotic balance of the cancer cells. We first analyzed mRNA expression of pro-apoptotic Bak and Bax along with that of anti-apoptotic Bcl-2 and Bcl-x_L, using breast cancer specimens of 27 patients. Bak mRNA was expressed much less in ERα negative breast cancers, along with reduced expression of Bcl-2. Immunostaining of sections of 108 patients confirmed the observation. Next, stable transformants of MCF-7 cells with sense Bak expression vector showed fewer colonies in soft agar compared with the parental cells, while stable introduction of antisense Bak vector enhanced colony formation at lower estradiol concentrations. The reduction of Bak may play important roles in malignant development of breast cancer to acquire estrogen independency, counteracting the reduced Bcl-2. *Cell Death and Differentiation* (2000) **7,** 439–446.

Keywords: breast cancer; Bak; apoptosis; estrogen receptor

Abbreviations: CT, charcoal treated; E_2, 17β- estradiol; ER, estrogen receptor; GAPDH, glyceraldehyde 3-phosphate deyhdrogenase; PRF, phenol red free; RT–PCR, reverse transcription polymerase chain reaction

Introduction

Apoptosis plays important roles in various biological events, such as morphogenesis, homeostasis, and carcinogenesis. Among numerous regulators of apoptosis identified so far, Bcl-2 related family members are thought to play a central role: anti-apoptotic members, represented by Bcl-2, and pro-apoptotic ones, such as Bax, are thus recognized as central regulators of apoptosis.[1,2] The Bcl-2 gene was first identified as a part of the fusion gene in t(14;18) translocation commonly found in follicular lymphoma.[3] Since then, Bcl-2 has been intensively investigated in various cancers and shown to be associated with poorer prognosis in prostate cancer, colon cancer, and neuroblastoma, in accordance with its anti-apoptotic nature.

In breast cancer, on the other hand, Bcl-2 expression has been reported to be associated with better outcomes in patients treated with either hormone- or chemo-therapy.[4] Joensuu *et al*. reported that moderate or high expression of Bcl-2 protein in breast cancer was associated with improved short-term (5 years) disease-specific free survival when compared with its weak, or non-, expression, although Bcl-2 was not an independent prognostic variable and there was no association with long-term survival.[5] Moreover, the absence of Bcl-2 expression was related to shortened periods of disease-free survival and overall survival in axillary node-positive breast cancer patients, but not in those without node metastasis.[6,7] These complicated findings in breast cancer might be explained by the notion that the expression of Bcl-2 has been significantly associated with several factors including estrogen receptor α (ERα) and p53, which also influence the malignancy of breast cancer.[4,6–8] In fact, Bcl-2 has been reported to be up-regulated by estradiol (E_2) in ERα-positive breast cancer cells MCF-7,[9,10] and down-regulated by p53.[11] The interaction between Bcl-2 and these factors, in particular ERα, is thus thought to play an important role in clinical characterization of breast cancer, since ERα is closely associated with the development of breast cancer.

However, the observation that increased expression of anti-apoptotic Bcl-2 is often associated with overexpressed ERα seems to be in contradiction to separate clinical observation that ERα-negative breast cancers, which express Bcl-2 at low levels, show more aggressive phenotypes and greater resistance to endocrine- and chemo-therapy than ERα-positive dose.[12] These findings suggest that, accepting the obvious link between reduced apoptosis of cancer and its malignancy, other regulators of apoptosis than Bcl-2 must be considered to evaluate the apoptotic status of breast cancer together with ERα.

Along these lines, several studies have been performed for the evaluation of other Bcl-2 family members, such as pro-apoptotic Bax in breast cancer. Reduced expression of Bax was reported to be associated with poorer responses to combination chemotherapy and with shorter survival in women with metastatic breast cancer,[13] although the

expression of Bax was not correlated with ERα status.[13,14] These suggest that Bax alone may not be a candidate for the determining factor in apoptosis regulation in ERα negative breast cancer.

Bak was first identified as a protein which interacts with an anti-apoptotic viral protein adenovirus E1B 19K, and it shares homology with other Bcl-2 family members within the well conserved BH1 and BH2 domains.[15–17] Bak induces apoptosis of various mammalian cells as well as yeast cells,[15–19] and reduced expression of Bak protein was found in primary colorectal adenocarcinomas and gastric adenocarcinomas compared with normal tissue,[20,21] indicating the possible involvement of Bak in these types of carcinogenesis. In breast cancer, no apparent difference of Bak expression was observed between cancerous and normal tissue,[22] although no reports with regard to an association between Bak expression and ERα status in breast cancer had appeared.

Thus, aiming to clarify the mechanisms of regulation and deregulation of apoptosis in breast carcinogenesis with regard to ERα status, we analyzed mRNA expression of pro-apoptotic Bak and Bax genes along with that of anti-apoptotic Bcl-2 and Bcl-x_L, using cancerous tissue from Japanese breast cancer patients. We found that both mRNA and protein expression of pro-apoptotic Bak in cancer tissue, as well as expression of Bcl-2 were less in ERα-negative breast cancers than in ERα-positive. Furthermore, *in vitro* experiments using MCF-7 cells showed that Bak was a factor participating in apoptosis regulation and thus altering the malignancy of the cells.

Our results indicate that the reduction of anti-apoptotic Bcl-2 and the increase of malignant phenotypes in ERα negative breast cancer can be in part explained, from the aspect of apoptotic balance, by the apparently coincidental reduction of pro-apoptotic Bak, suggesting that the reduction of Bak may play some roles in the malignant development of breast cancer, especially in the conversion to acquire estrogen independency.

Results

Expression of Bcl-2, Bcl-x_L, Bak, and Bax mRNA in primary breast cancer

It is still unknown what it is that actually maintains the apoptotic balance in ERα negative breast cancer, where the expression of anti-apoptotic Bcl-2 decreases in spite of its malignant phenotypes. To explore the possible involvement of Bcl-2 family members in breast carcinogenesis with special reference to ERα status, mRNA expression of Bak, Bax, Bcl-2, and Bcl-x_L, along with ERα target gene pS2, were analyzed in surgical specimens of 27 primary breast cancers. The representative pattern of their expression, measured by RT–PCR, is shown in Figure 1. The expression levels were quantified by autoradiography and normalized against the internal control GAPDH expression. We first compared mRNA expression by ERα status, and, as shown in Figure 2, the mean expression of Bcl-2 in ERα positive breast cancer was much higher than that in ERα negative, confirming previous observations.[4,8] In addition, pro-apoptotic Bak and Bax expression was 4.1-fold and 3.0-fold higher in ERα positive cancer than in ERα negative, respectively. On the other hand, expression level of another anti-apoptotic, Bcl-x_L, showed no significant difference between ERα positive and negative cancers.

Next, we studied how closely the expression of these genes is regulated by ERα, in terms of the correlation with ERα protein levels measured by EIA and mRNA expression of pS2, a primary target gene of ERα, among the 27 breast cancer specimens. As reported previously, anti-apoptotic Bcl-2 showed a significant and positive correlation with ERα-EIA values (correlation coefficient r=0.48, P=0.02) or pS2 (r=0.67, $P<0.001$). Interestingly, a strong correlation of Bak mRNA expression and ERα protein levels was observed (r=0.57, $P<0.01$), and this was confirmed by the good correlation between Bak and pS2 (r=0.42, P=0.03). Expression of Bax mRNA was also correlated with ERα protein levels (r=0.43, P=0.03), though no significant correlation with pS2 was observed (P=0.3), indicating that Bax may not be functionally associated with ERα. In contrast to these genes, expression of Bcl-x_L showed no apparent correlation with either ERα or pS2 (P=0.5 or P=0.4, respectively). Our measurement of mRNA expression by RT–PCR was confirmed by the correlation of measured ERα with ERα protein (r=0.65, $P<0.001$).

These data indicate that, in accordance with anti-apoptotic Bcl-2, pro-apoptotic Bak may participate in the development of breast cancer.

Expression levels of the Bcl-2 family genes may be related to several clinical parameters of breast cancer. In fact, we found a positive association between Bak expression and clinical staging: Mean Bak expression

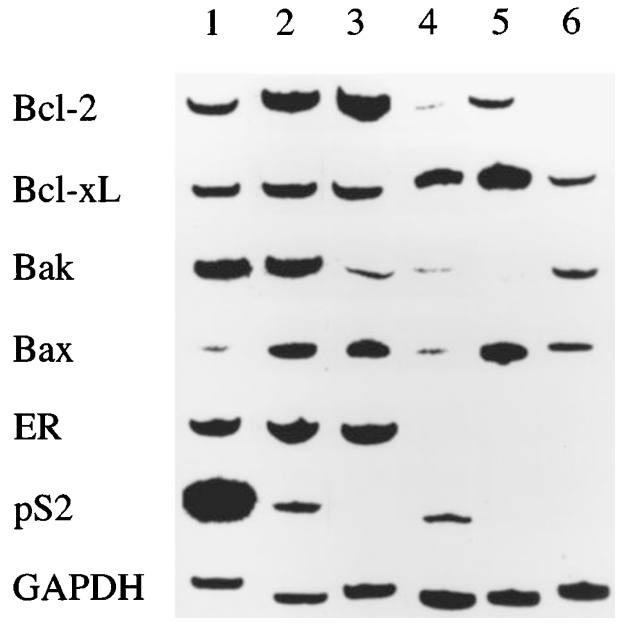

Figure 1 Expression of Bcl-2, Bcl-x_L, Bak, Bax, pS2, and ERα mRNAs in breast cancer tissues by RT–PCR analysis. One μg of total RNA prepared from the resected tissue of breast cancer was reverse transcribed and then subjected to the PCR to detect indicated gene expression as described in Materials and Methods. Each lane shows the results from the same patients

was 2.5-fold higher ($P<0.05$) in breast cancer with stage 2 or more (n=16) than that with stage 1 (n=7), excluding non-invasive cancer. On the other hand, Bcl-2, Bcl-x_L, and Bax were not correlated with clinical staging.

Immunohistochemical staining of primary breast cancer with anti-Bak antibody

We then carried out immunohistochemical staining of breast tissues resected from primary breast cancer patients with anti-Bak monoclonal antibody. The first step was to determine which cells express Bak protein, and Figure 3 shows a typical staining of breast cancer tissue with normal mammary gland: Both normal epithelial cells (white arrow heads) and cancerous regions (black arrow heads) express Bak, while other components were not stained by Bak antibody, indicating that the observed Bak mRNA expression was derived mainly from cancerous cells mixed with minor contaminated normal breast epithelial cells. Next, we examined Bak protein expression among breast cancer tissue sections of 106 patients, and we found that normal breast epithelial cells were always stained intensely, while Bak immunostaining of cancerous regions was consistently cytoplasmic, of varying intensity and frequently heterogeneous. The stained samples were categorized into three groups by staining grades as described in Materials and Methods, and the results are shown in Table 1 with ERα status: expression of Bak was significantly associated with ERα status (P=0.005), confirming our observation of the mRNA expression using RT–PCR analysis. Taken together, the expression of both protein and mRNA of Bak were significantly reduced in ERα negative breast cancers, compared to those in ERα positive, suggesting the possible involvement of Bak in the maintenance of apoptotic balance with Bcl-2 in breast cancer.

Table 1 Comparison of Bak staining levels with ERα status in primary breast cancer

	Bak staining levels		
Erα status	**1**	**2**	**3**
Positive	10	20	27
Negative	20	19	10

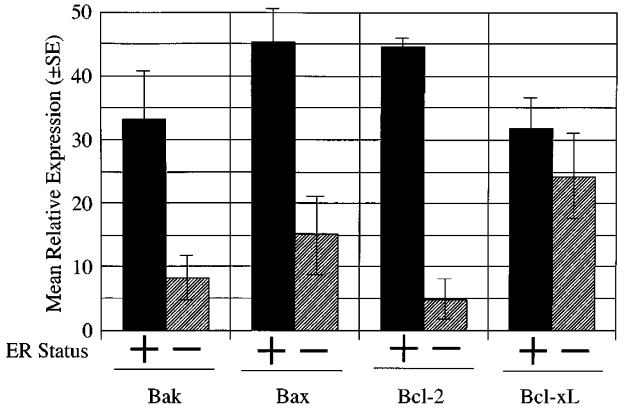

Figure 2 Comparison of the mRNA expression of Bcl-2 and Bak in ERα-positive and ERα-negative breast cancers. Relative expression levels of Bcl-2 and Bak measured using RT–PCR were quantified with autoradiography and normalized against GAPDH expression. Mean relative expression of the genes were shown by column. Bars indicate standard errors. Statistical analysis was performed by the Mann-Whitney's *U*-test

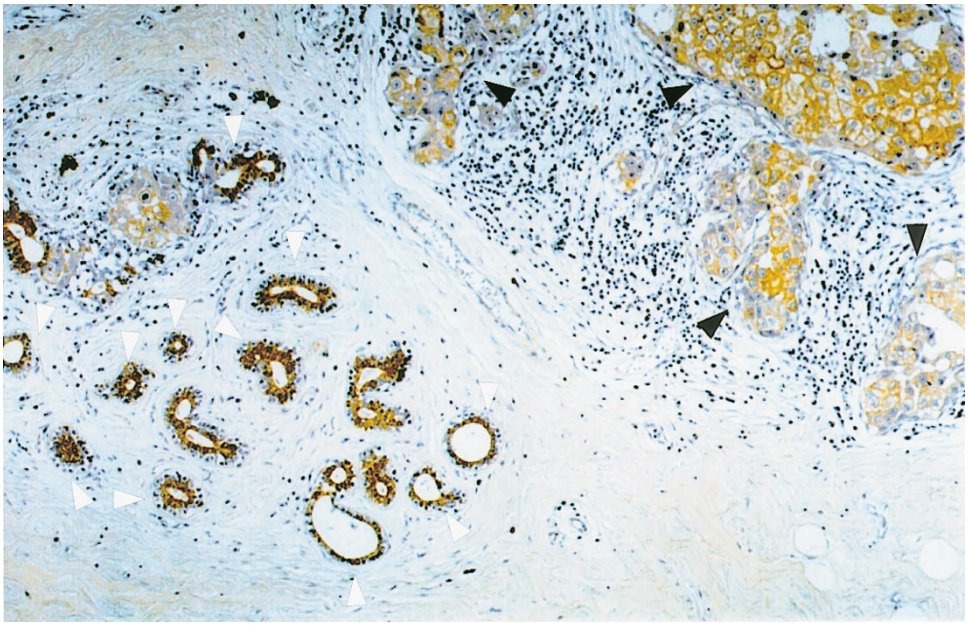

Figure 3 Immunostaining of breast cancer tissue section with anti-Bak antibody. Paraffin embedded sections of breast cancer tissues resected from the breast cancer patients were immuno-histochemically stained with anti-Bak monoclonal antibody as described in Materials and Methods. White arrow heads indicate the normal mammary epithelium. Black arrow heads, breast cancer. Bak was stained in the cytoplasma of both tumor cells and normal mammary epithelial cells

These data led us to study further the role of pro-apoptotic Bak in the regulation of apoptotic balance in breast cancer with regard to ERα status.

Constitutive expression of Bak protein in MCF-7 cells

Since both mRNA and protein levels of Bak were reduced in ERα negative breast cancers compared with ERα positive, we next studied whether forced expression of Bak could alter the growth of breast cancer cells. To assess the effects of constitutive Bak expression on cell growth, we established several stable transformants of MCF-7 cells using Bak expression vector. The established clones, MCF-7/BAKS #4, #7, #10, and #15 showed 3–5-fold expression of Bak, compared with the parental MCF-7 cells (Figure 4a). Together with parental MCF-7 cells, four independent transformants were subjected to soft agar colony formation assay. The assay was performed in the presence of 0.1, 1 or 10 nM of E_2, which corresponds to a physiological E_2 concentration in the plasma. As shown in Figure 4b, all transformants showed decreased numbers of colonies compared with parental MCF-7 cells. Especially at 0.1 nM of E_2, Bak-expressing transformants formed very few colonies. Thus, only 3–5-fold constitutive expression of Bak was found to be sufficient for the suppression of tumorigenicity of MCF-7 cells at various E_2 concentrations. The partial recovery of the growth of transformants by extended addition of E_2 could be accounted for, at least in part, by the induction of anti-apoptotic Bcl-2 protein to counteract the pro-apoptotic action of Bak.

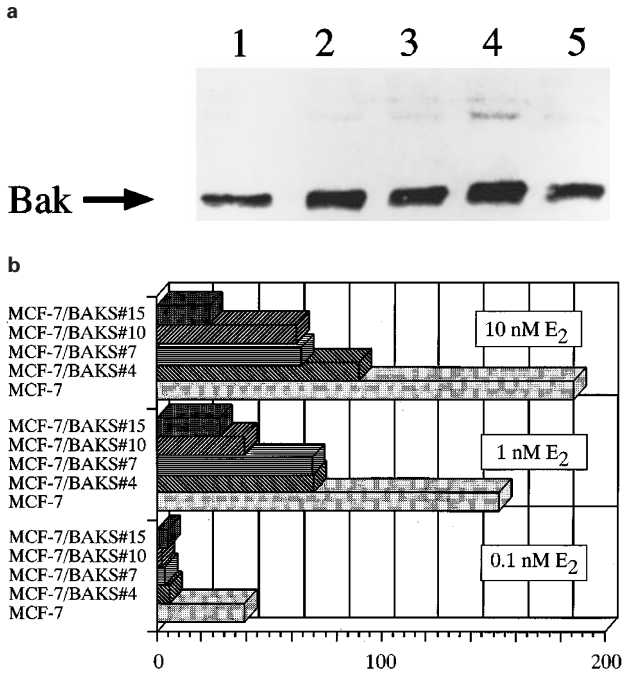

Figure 4 Stable transformants of MCF-7 cells with Bak expression vector. MCF-7 cells were stably transformed with Bak expression vector. After 2 weeks of culture in the presence of 500 μg/ml of G418 to select colonies, individual colonies were isolated and further cultured in the presence of 200 μg/ml of G418. (**a**) Western blot analysis of the expression of Bak protein in parental and transformed MCF-7 cells. Cell lysates of the cells were prepared and 1 mg each of the extracts were immunoprecipitated with rabbit anti-Bak polyclonal antibody at 4°C overnight. The precipitates were loaded onto 15% of SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane. Lane 1, parental MCF-7 cells; Lanes 2–5, MCF-7/BAKS #4, #7, #10, and #15, respectively. The Bak protein was visualized using goat anti-Bak polyclonal antibody and Amplified ImmunAP kit as described in Materials and Methods. (**b**) Anchorage independent cell growth assay of MCF-7 transformants with Bak expression vector under various concentrations of E_2. Stable transformants of MCF-7 cells with pCMVBAKS vectors were maintained in PRF-RPMI/10% CT-FCS with 1 nM of E_2 for 1 week. Cells were transferred onto the soft agar medium with indicated concentrations of E_2 as described in Materials and Methods. After 2 weeks culture, the numbers of colonies bigger than 300 μm appeared were counted

Stable transformants of MCF-7 cells with Bak antisense vector

We next evaluated the contribution of endogenous Bak to the regulation of cell growth when ERα signaling is suppressed: whether altered expression of Bak protein would affect on the growth of breast cancer cells under a very low E_2 condition, where Bcl-2 expression is reduced. Several stable transformants of Bak antisense expression vector were established. Western blot analysis of Bak protein in parental MCF-7 cells as well as transformants, MCF-7/BAKAS #21 and #22 is shown in Figure 5a. Since MCF-7 cells were sensitive to E_2 depletion, the colony formation assay for the Bak antisense expressing transformants was performed under a low E_2 condition. The transformants formed colonies larger than the parental MCF-7 cells (Figure 5b), and they also showed increased numbers of colonies (approximately twice, $P<0.05$), regardless of E_2 concentration (Figure 5c). These results indicate that endogenous expression of Bak in MCF-7 cells is indeed involved in the regulation of cell growth under a very low E_2 condition, and that decreased expression of Bak itself can modify the tumorigenicity of breast cancer.

Discussion

In this study, we revealed that both mRNA and protein expression of pro-apoptotic Bak in ERα negative breast cancer, which shows aggressive phenotypes, were significantly reduced compared with those in ERα positive; there was also a decrease in pro-apoptotic Bcl-2, suggesting a possible involvement of Bak gene in the malignant conversion of breast cancer.

Since Bak and Bax share their amino acid sequence identity[15–17] and the ability to heterodimerize with anti-apoptotic Bcl-2 and Bcl-x_L, inducing apoptosis,[15–19] Bak may also be a tumor suppressor in some cancers. Though no mutations have been reported so far, reduced expression of Bak has been commonly observed in primary colorectal and gastric adenocarcinomas in the early stages,[20,21] indicating the possible involvement of Bak in the early stages of these types of carcinogenesis. Recently, Zapata *et al*. reported the expression of Bcl-2 family members in breast cancer cell lines and primary tumors.[22] We observed no significant differences in Bak protein expression between normal mammary epithelium and breast cancer (Saji *et al*. unpublished data), suggesting

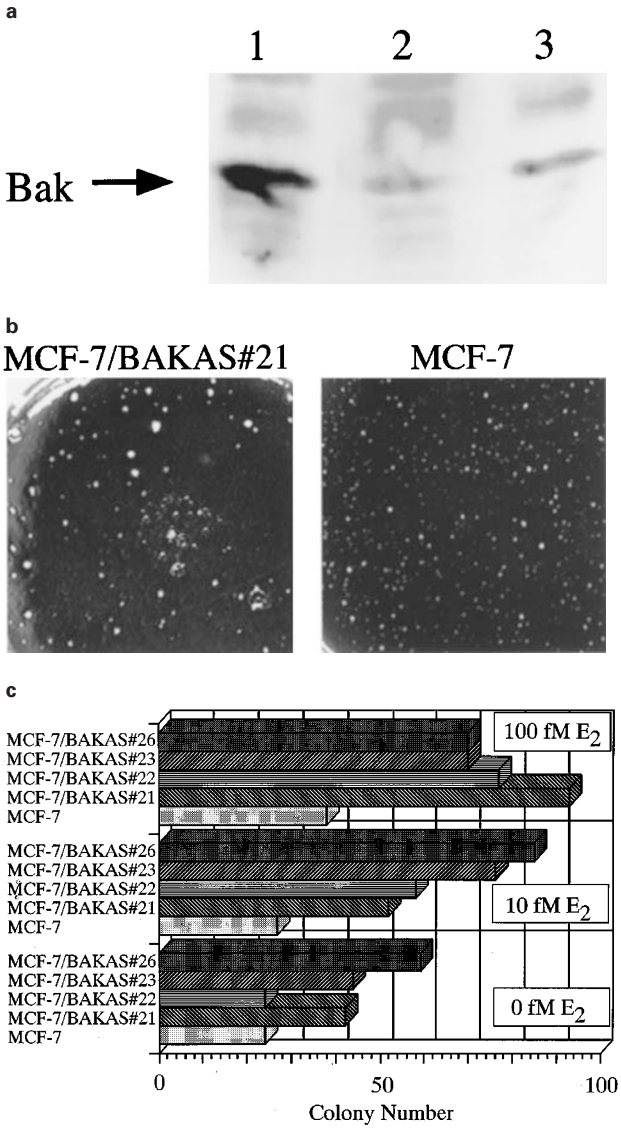

Figure 5 Stable transfectants of MCF-7 cells with Bak antisense expression vector. Stable transformants of MCF-7 cells with Bak antisense expression vector were established as described in Materials and Methods. (**a**) Western blot analysis of the expression of Bak protein in parental and transformed MCF-7 cells. Fifty μg each of the extracts were loaded onto 15% of SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane. Lane 1, parental MCF-7 cells; Lanes 2 and 3, MCF-7/BAKAS #21 and #22, respectively. The Bak protein was visualized using goat anti-Bak polyclonal antibody and Immun-Star substrate as described in Materials and Methods. (**b**) Microscopy of the colonies of MCF-7 and MCF-7/BAKAS #21 cells appeared on soft agar. The cells were grown on soft agar culture for 2 weeks without supplement of E_2. (**c**) Anchorage independent cell growth assay of MCF-7 cells stably transformed with Bak anti-sense vector. The stable transformants of MCF-7 cells were maintained in PRF-RPMI/10% CT-FCS without supplement of E_2 for 1 week. Cells were then transferred onto the soft agar medium with indicated concentrations of E_2 as described in Materials and Methods. After 2 weeks culture, the numbers of colonies bigger than 100 μM appeared were counted

that, in contrast to Bax, Bak is not involved in the early stage of breast carcinogenesis. On the other hand, we found that Bak expression decreased in ERα negative breast cancers appeared which show more aggressive phenotypes than ERα positive, emphasizing the point that the reduction of Bak occurs in the malignant development of breast cancer, i.e., reduction of Bak is associated with the conversion to hormone-independent cancer.

Enhanced expression of Bak reduced colony formation activity even in a high E_2 condition, while the reduction of Bak by the introduction of antisense vector provided a growth advantage to the MCF-7 cells in a low E_2 condition. This, then, is the first report to show that reduction of the pro-apoptotic factor alters the tumorigenicity of breast cancer cells.

It has been shown that the expression of Bak protein as well as mRNA was reduced by the E_2 supplementation in MCF-7 cells,[23] indicating that Bak may also be regulated by E_2 in breast cancer. On the other hand, we observed a positive correlation between expression of Bak and ERα in this study; a reporter assay using 5′ flanking region of Bak gene showed an increased promoter activity by E_2 supplementation in MCF-7 cells (Eguchi H unpublished data). In fact, Leung and Wang have reported that the suppression of Bak expression in MCF-7 cells occurred after 72 h treatment with E_2, while Bcl-2 responded even after 24 h treatment.[23] These data imply the possibility of some other mechanisms of regulating Bak gene expression other than ERα-E_2 signaling in breast cancer.

If the expression of Bak were maintained at high levels even in ERα negative breast cancer, where the expression of Bcl-2 was low, the balance between Bak and Bcl-2 would lead to a more apoptotic situation. However, in ERα negative breast cancers, we found decreased expression of both Bcl-2 and Bak, maintaining a balance of these factors. These data indicate that Bak may be one of the factors involved in the mechanisms of the adaptation to survive in a low E_2 condition, especially when ERα expression is lost in breast cancer, resulting in a reduction of Bcl-2. Thus, the reduction of Bak expression is thought to provide a growth advantage in the loss of ERα during the progression of breast cancer.

The location of Bak gene has been recently assigned at 6p12.3;[24] but several studies using LOH (loss of heterozygosity) analysis of breast cancer have been done, none has so far mentioned this site as common deleted region.[25] This indicated that decreased expression of Bak is not due to the chromosomal deletion of Bak gene, which suggests the possibility of enhancing Bak expression by activating some transcriptional factors required for Bak gene expression, or by changing the chromatin structure around the Bak gene promoter region.

Recently another subtype of estrogen receptor, ERβ was identified.[26] Although we observed much lower levels of ERβ expression in breast cancer than those of ERα (data not shown), it would be of great interest to study the possible role of ERβ in regulation of apoptosis in breast cancer cells, specifically ERα negative ones.

Since overexpression of Bak induces apoptosis of cells, including MCF-7 cells, as effectively as Bax does (Eguchi *et al*. unpublished data), Bak may be an alternative candidate as a target gene for the treatment of ERα negative breast cancer. In ERα negative breast cancers, not only ERα is absent, resulting in resistance to endocrine therapy, but tumor suppressor gene p53 is also frequently

mutated or deleted. Since the pro-apoptotic Bax gene is induced by chemotherapeutic reagents or X-ray irradiation mainly via p53, curing ERα negative breast cancer remains a formidable task. Ossina *et al*. have shown that expression of Bak is directly induced by treatment with interferon γ in human colon adenocarcinoma cell line HT-29, with Bax remaining unchanged[27] which strongly indicates that the transcriptional regulation of Bak is different from that of Bax, and possibly independent of the p53 pathway. Thus, understanding the molecular mechanisms of transcriptional regulation of Bak gene may provide new insight into therapy for ER negative breast cancer with inactivated p53.

In summary, we found reduced expression of Bak in ERα negative human breast cancers, compared with that in ERα positive. *In vitro* experiments using MCF-7 cells indicated that the reduction of Bak provides a growth advantage to the cancer cells, especially in a low E_2 condition. These data suggest that Bak may have potential as a tumor suppressor in breast carcinogenesis, and, further, that decreased expression of Bak may be one of the important events in the breast cancer cell's adaption toward hormone independence.

Materials and Methods

Tissue samples for analysis of mRNA expression

A total of 27 study subjects were randomly selected from the RNA/DNA collection of our breast cancer research project in Saitama Cancer Center as described previously.[28,29] In this project, all possible surgical specimens of primary breast cancer were collected and immediately subjected to isolation of RNA and DNA.

Cell culture and total RNA isolation

MCF-7 cells were maintained in RPMI medium supplemented with 2 mM L-glutamine, 10% FCS (fetal calf serum) and 2 μg/ml of gentamicin and grown in the presence of 5% CO_2 in air at 37°C. For some experiments, the cells were transferred in phenol-red free (PRF) RPMI medium (Life Technologies, Rockville, MD, USA) with 5% FCS treated with charcoal and dextran T-70 (Amersham Pharmacia Biotech, UK) (CT-FCS) to deplete steroids, in the presence or absence of 1 nM estradiol (E_2). Total RNA was prepared from the cells by the method of Chomczynski and Sacchi.[30]

Quantification of Bcl-2, Bcl-x_L, Bak, Bax, ER, pS2 and GAPDH

Quantitative RT–PCR (reverse transcription-polymerase chain reaction) was performed using the RNA PCR kit (AMV) Ver. 2 (TAKARA, Tokyo, Japan) essentially as previously described.[31] Oligonucleotides used in PCR amplficiation were as follows: BCL03, GAG TTC GCC GAG ATG TCC AG, and BCL04, TCA CTT GTG GCT CAG ATA GG for Bcl-2;[3] BCX01, AGT TTG AAC TGC GGT ACC GG, and BCX02, GCA TTG TTC CCA TAG AGT TC for Bcl-x_L;[32] BAK01, ACG CTA TGA CTC AGA GTT CC, and BAK02, CTT CGT ACC ACA AAC TGG CC for Bak;[15–17] BAX03, ATG GAC GGG TCC GGG GAG CA, and BAX04, CCC AGT TGA AGT TGC CGT CA for Bax;[33] GAP1, ACA TCG CTC AGA CAC CAT GG, and GAP2, GTA GTT GAG GTC AAT GAA GGG for GAPDH.[34] The oligonucleotides, PS1 and PS2 for pS2 detection were described previously.[35] The prepared RNA (1 μg) was reverse transcribed using random nanomers and AMV reverse transcriptase at 55°C for 30 min. Two μl of the synthesized cDNA was subjected to PCR amplification with 200 ng each of specific primers and 3 μCi of [α-^{32}P]dCTP using *Taq* polymerase (TAKARA). PCR comprised 24 cycles for GAPDH, 25 cycles for Bcl-x_L and Bak, 27 cycles for Bcl-2, and 27 cycles for pS2, with denaturing at 95°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 1 min in each cycle using a *GeneAmp*™ PCR System 9600 (PE Applied Biosystems, Foster City, CA, USA). In the case of Bak, *Pfu* DNA polymerase was substituted for *Taq* polymerase, and the total RNA was treated with RQ1 RNase free DNase (Promega, Madison, WI, USA) to eliminate contaminated DNA prior to the reverse-transcription reaction. The PCR products were applied onto 5% acrylamide gel electrophoresis, and radioactivity was quantified by autoradiography with Fuji Bio-Image Analyzer BAS2000 (Fuji film, Tokyo, Japan). Quantification of ERα was performed as described previously.[28] The linearity of the quantification of RT–PCR products was ascertained by the experiment using varied amounts of total RNA. The PCR products labeled by incorporated α-^{32}P-dCTP were linearly increased until 1 μg of total RNA from breast cancer cell lines as described previously.[29]

ERα protein assay

ERα enzyme immuno-assay for tumor samples was performed with ER-EIA kits (Abbott Laboratories, Abbott Park, IL, USA). ERα values <5 fmol/mg protein were considered as negative.

Immunohistochemical study of Bak expression

One-hundred and six records of primary breast cancer patients who had received mastectomy from 1994–1996 in Tokyo Metropolitan Komagome Hospital were included in this study. The 3–5 μm sections of paraffin-embedded primary breast tumor tissues were applied to indirect anti-peroxidase immunohistochemical assay, StrepABC technique (Dako, Carpinteria, CA, USA), for assessing the expression of Bak. Analysis of Bak staining was carried out using an anti-Bak monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:100 dilution, incubated overnight after citrate buffer microwave pre-treatment. Evaluation of expression was analyzed in the five discrete areas of the tumor showing the greatest accumulation, and the values represent the mean of the areas measured. Immunopositive normal breast ducts were used as internal positive controls. Semi-quantitative estimation of expression was done by assessing the percentage of stained tumor cell cytoplasms, which was classified into three categories (0–5, 5–50, and >50%). All immunohistochemical and pathological assessments were conducted by two investigators under completely blind conditions.

Statistical examination

Correlations of mRNA expression of Bcl-2, Bcl-x_L, Bak, or Bax with that of pS2 and ERα-EIA values were evaluated by Spearman's correlation coefficients. The statistical significance of differences of the mRNA expression levels of Bcl-2, Bcl-x_L, Bak, Bax, pS2, and ERα by ERα status were determined by the Mann-Whitney's *U*-tests. Association of Bak immunostaining levels with ERα status was assessed by the chi-square-based measures in a cross-classification.

Preparation of stable transformants of MCF-7 cells with sense or antisense Bak expression vector

Full coding sequence of Bak cDNA prepared using proof-reading PCR was inserted into pRC/CMV vector (Invitrogen, Carlsbad, CA, USA)

driven by CMV (cytomegalovirus) promoter in both directions. The orientation of the inserts was determined by sequencing with A.L.F.II automated sequencer (Amersham Pharmacia Biotech, UK). To establish stable transformants, MCF-7 cells were transfected with sense or antisense Bak expression vector using lipofectin (Life Technologies, Rockville, MD, USA) as the manufacturer recommends. After 1 day culture in RPMI supplemented with 10% FCS, the cells were grown in RPMI medium supplemented with 10% FCS containing 500 μg/ml of G418 for 2 weeks. Isolated colonies were trypsinized in metal ring cups, and the cells were further cultured in the presence of 200 μg/ml G418.

Anchorage independent growth of parental MCF-7 and Bak expressing transformants

Cells (10 000) in 1.5 ml of phenol red-free (PRF)-RPMI, 0.3% agar, 5% charcoal-treated (CT)-FCS were exposed to various concentrations of E_2 over 4.0 ml of PRF-RPMI, 0.5% agar, 5% CT-FCS containing the same concentration of E_2. Culture was incubated at 37°C in 5% CO_2 for 2 weeks. On day 14, the number of colonies per dish was counted.

Western blot analysis

Rabbit (sc-832) and goat (sc-1035) antibodies against human Bak were purchased from Santa Cruz. Amplified AP ImmunoBlot kit was from Bio-Rad Laboratories (Hercules, CA, USA). Parental as well as stable transformants of MCF-7 cells were washed once with 1 × PBS (−), and then lysed with lysis buffer (10 mM Tris-HCl (pH 8.0), 1% sodium deoxycholate, 0.5% Triton X-100, 0.1% SDS, 100 μg/mL phenylmethanesulphoyl fluoride (PMSF), and leupeptin). Lysed cells were centrifuged at 13 000 r.p.m. for 30 min, then the cleared lysate was stored as a cell extract. Protein concentration of the cell extracts was determined using BCA Protein Assay Reagent (PIERCE, Rockford, IL, USA). For Bak sense vector transfectants, 1 mg of each cell extract was precipitated with rabbit anti-Bak polyclonal antibody and protein A agarose at 4°C overnight. The precipitates were subjected to a 15% SDS–PAGE. Proteins were electrically transferred to a nitrocellulose membrane (Bio-Rad). The membrane was briefly washed with 1 × TBS (Tris-HCl, NaCl), and then blocked with 5% non-fat milk in TTBS for 2 h at room temperature. After washing twice with TTBS for 10 min, the membrane was incubated with goat anti-Bak antibody solution (1/1000 dilution in TTBS) overnight at room temperature. The membrane was washed twice with TTBS, and reacted with second antibody solution (1/3000 dilution of biotinylated goat anti-rabbit IgG in TTBS) for 2 h, and then washed twice with TTBS. The membrane was incubated with streptoavidine-biotin complex for 1 h, then washed twice with TTBS, followed by TBS. Finally, the membrane was incubated in alkaline phosphatase reaction mixture for 30 min at room temperature. For Bak antisense transfectants, 60 μg each of cell extracts were directly subjected to the SDS–PAGE, followed by Western-blotting analysis. The transferred membrane was incubated with 1/3000 diluted goat antibody against Bak for 5 h, 1/3000 diluted biotinylated anti-goat IgG (sc-2042) for 1 h, and 1/2000 diluted anti-biotin-AP Fab-fragments (Boehringer Mannheim, GmbH, Germany) for 1 h. Detection of Bak was performed using Immun-Star Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and Fuji Luminoimage Analyzer LAS-1000 (Fujifilm, Tokyo).

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