

BCL-6 Prevents Mammary Epithelial Apoptosis and Promotes Cell Survival

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Abstract

Objective: Proliferation, differentiation and apoptosis are essential processes in the normal functions of the mammary epithelium. The hypothesis examined in this study is that BCL-6 is critically important not only for regulating B cell growth and development, but also for influencing mammary epithelial apoptosis.

Methodology: Full length BCL-6 cDNA was retrovirally transduced into EpH-4 cell line. We used flow cytometry of BrdUrd-stained cells to investigate the cell cycle duration of control and transduced cells. TUNEL was used as a marker of apoptosis to find out differences in the frequencies of apoptotic cells in control and transduced cells.

Results: Restoration of human BCL-6 into EpH4 cells not only inhibits apoptosis, but also prolongs the cell cycle. The results also indicated that the cell cycle time of BCL-6-transduced EpH4 cells was prolonged by about 3 hours. We found differences in the frequencies of viable and apoptotic cells in cultures of the parent EpH4 cells, control transduced EpH4 cells and BCL-6-transduced EpH4 cells.

Conclusion: The results suggest that BCL-6 may be involved in mammary growth or mammary gland cell kinetics, but not in mammary gland development (JPMA 58:494;2008).

Introduction

The human breast contains about twelve main ducts that converge in the nipple, branching interlobular ducts and alveoli. Three epithelial components can be identified in the functional mammary gland: the luminal epithelium, the alveolar epithelium and the myoepithelium. The growth regulation of this gland requires a close coordination between proliferation, differentiation and apoptosis. These processes are driven by several signals including: hormonal signals, milk stasis and the extracellular matrix (ECM). There is an extensive remodeling of the ECM during involution. Therefore, ECM can be seen as an integrator of function that positively maintains the differentiation state or on the other hand regulate apoptosis and development of cancer.

Among the most important signaling pathways is the Signal Transducers and Activators of Transcription (STAT). STAT proteins are a family of cytoplasmic transcriptional regulators activated by cytokines and growth factors. Of relevance to mammary gland physiology, STAT5 is specifically activated by prolactin during lactation. STAT5, therefore, activates transcription of milk protein genes.^{1,2} Preliminary reports suggested that STATs may be involved in other hormonal signaling systems which are relevant to mammary gland physiology and tumorigenesis such as the insulin-like growth factor.

Reljic et al demonstrated that the zinc finger transcription factor BCL-6 is highly expressed in the germinal center B cells.^{3,4} BCL-6 was first cloned in human B cell lymphoma.^{5,6} BCL-6 is essential in the normal humoral

immune response as demonstrated by the lack of germinal centers in mice, which have homozygous disruptions in this gene.⁷ BCL-6 protein expression was found outside the lymphoid system, various epithelial sites, keratinocytes of mature animals and in various skin tumours.^{8,9} Additionally, Bos and colleagues showed that BCL-6 is expressed in breast cancers.¹⁰ This study aimed to investigate the expression of BCL-6 in mammary epithelial cells and to determine whether this expression; if it occurred, is important in the maintenance of cell viability and prevention of apoptosis or not. We show that BCL-6 is expressed in mammary epithelial cells suggesting that it is important in maintaining cell viability and preventing apoptosis.

Materials and Methods

Eph4 cells are maintained routinely in our laboratory. 3.5x10⁵ cells were seeded on 60mm plates which had been previously coated with 0.4ml of EHS-ECM (Sigma). The MEM medium was replaced after growing in 10% FCS for 24 hours by serum free medium with prolactin 3 g/ml (Sigma), hydrocortisone 1 g/ml (Sigma) and insulin 5 g/ml (Sigma). Medium and growth factors were exchanged every two days for a week and incubated at 37°C in humidified 5% CO₂ in air.

Full length human BCL-6 cDNA was subcloned into the retroviral shuttle vector pBabe containing a puromycin resistance gene (pBP). pBP plasmid cDNA containing the BCL-6 fragment was purified from *Escherichia coli* using the Miniprep DNA purification kit (Qiagen, Sussex, UK) as per the manufactures's

instructions. The presence and orientation of the BCL-6 fragment was confirmed by PCR. pBP and pBP containing BCL-6 (pBP-BCL-6) were transfected into the GP-E-86 ecotropic producer cell line using a calcium phosphate transfection kit (Invitrogen, Paisley, UK). Untransfected and transfected GP-E-86 producer cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS) and incubated at 37°C. After 24 hours incubation, the medium was changed and they continued growth at 37°C. NIH-3T3 cells were infected with supernatant containing retrovirus to determine its infectivity and titer. A transwell coculture method was used for retroviral-mediated gene transfer. Briefly, GP-E-86 producer cells containing pBP-BCL-6 or pBP and untransfected GP-E-86 cells were placed in the wells of 6-well plates (Costar) until they were 80% confluent. Then, transwells were inserted into the wells. Eph4 cells were plated at 2x10⁵ per well in a transwell coculture. Packaging cell line containing the BCL-6 and untransfected packaging cell line were placed in the wells of 6-well plates (Costar) and centrifuged 2200rpm for 90 minutes at 32°C with 4 g/ml of polybrene (Sigma).

For Flow Cytometry, 10⁶ cells were washed in PBS and plated in 10ml of MEM with 1% EHS-ECM, prolactin 3 g/ml (Sigma), hydrocortisone 1 g/ml (Sigma) and insulin 5 g/ml (Sigma) for 24 hours. The medium was then replaced by MEM with prolactin 3 g/ml (Sigma), hydrocortisone 1 g/ml (Sigma) and insulin 5 g/ml (Sigma) and 10 M BrdU. After a further 24 hours the cells were removed by trypsin, washed by PBS, fixed with 2% paraformaldehyde for 20 minutes and then permeabilised with 0.1% saponin in PBS/10% FCS. Cells were then digested with DNase (Invitrogen) for 1 hour at 37°C. Subsequent to incubation with PE conjugated anti-BrdU (Pharmingen), the percentage of positive cells was measured on FACS machine.

Determination of apoptotic cells was done by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL). 10⁶ cells were plated without serum, and with additives as above.

Eph4 cells were suspended in buffer and mixed with equal volumes of 8% paraformaldehyde for 10 min. The cells were pelleted at 1800 rpm for 5 min then resuspended in Dulbecco's MEM (Gibco) at a concentration of 2x10⁵/ml and 100 l volumes cytospun on to cleaned microscope slides at 450 rpm for 10 min (Shandon Cytospin 2; Shandon, Pittsburgh, PA, USA). Slides were air dried overnight, rehydrated in TBS for 15 min at room temperature (RT) and dried. The cells were covered by a 5 ml droplet of protein K diluted 1:100 in 10mM Tris (pH 8), incubated 5 min at RT then dipped

three times into TBS and dried. The specimen was covered with 100 l of supplied equilibration buffer and incubated for 30 min at RT. Excess buffer was poured off and freshly prepared TdT labelling mixture (3 ml TdT enzyme in 57 ml TdT labelling reaction mix (Frag EL:Calbiochem, Nottingham, UK) was layered on to the cells. The slide was incubated at 37°C in humidified chamber for 1.5 hours then washed 3 times in TBS at RT. A coverslip was applied over mounting medium (Frag EL) and sealed with nail varnish to prevent evaporation. At least 500 cells from randomly selected fields were scored by fluorescent light microscopy (494 nm). Viable cells stained blue whilst apoptotic cells appeared as small fragmented bodies staining bright green.

Results

According to the work published previously by Reljic et al³, we assume that any dysregulation of BCL-6 might lead to disruption of differentiation and/or apoptosis of mammary epithelium. We approach this question by using a non-tumour cell line Eph4. This cell line is able to differentiate i.e. by production of milk protein -casein. Therefore, withdrawal of serum may not only disrupt differentiation but also induce apoptosis.

Following the two-week selection period of culture in puromycin, transduced Eph4 cells were maintained in puromycin-containing medium. The parent cell line was maintained in the absence of puromycin. Eph4 cells transduced with empty vector, proliferated in liquid culture with the same growth kinetics as the parent cell line. However, Eph4 cells transduced with BCL-6 grew much more slowly (Figure 1). Expression of BCL-6 by the transduced Eph4 cells was confirmed by Western blot analysis (Figure 2). Our results show that the restoration of BCL-6 in Eph4 cell line not only inhibits cell cycle arrest and but also prolongs G1 phase in the cell cycle by approximately 3 hours. This finding is supported by the observation of increased cell size and protein content in the Eph4 cells expressing

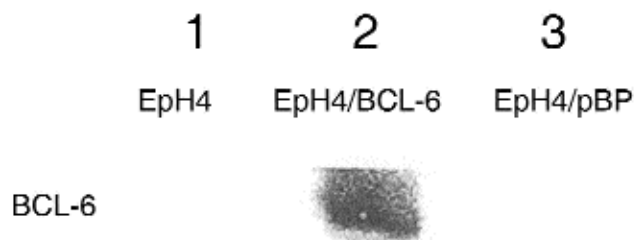


Fig 1: Eph4 cells transduced with BCL-6 or empty vector were grown in medium containing puromycin. Untransduced cells were grown in medium without puromycin. Eph4 cells transduced with BCL-6 proliferate much more slowly compared to those parent cell line and those transduced with empty vector.

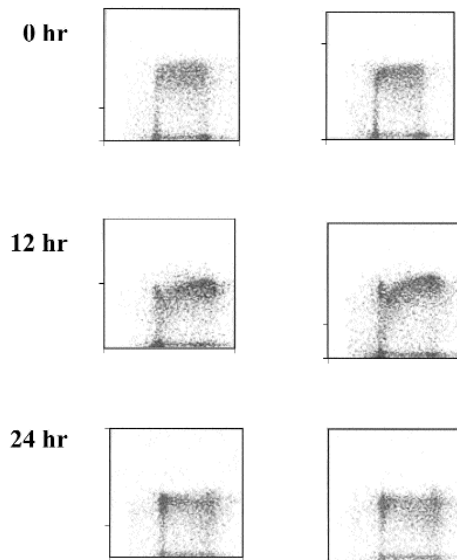


Fig 2: Western analysis showing BCL-6 expression in Eph4. A photograph of retrovirally transduced Eph4 after selection. BCL-6 expression in Eph4 infected with BCL-6 and empty retroviruses. 25 g of (RIPA lysate) was loaded in each lane and run on 7.5% SDS-PAGE. Expression of BCL-6 by the transduced Eph4 cells was shown and confirmed by Western blot analysis. Four independent set of experiments were carried out. Results shown are representative of four experiments.

transduced BCL-6.

Restoration of BCL-6 into Eph4 cells not only inhibits apoptosis, but also prolongs the cell cycle and results in increased cell size and protein content. We then used flow cytometry of bromodeoxyuridine (BrdUrd)-stained cells to investigate the cell cycle duration of the control and transduced cell lines. Specimen results are shown in Figure 3. At time 0 hours, the BrdUrd labeled S phase cells are identifiable by their green fluorescence. The cells in G1 and

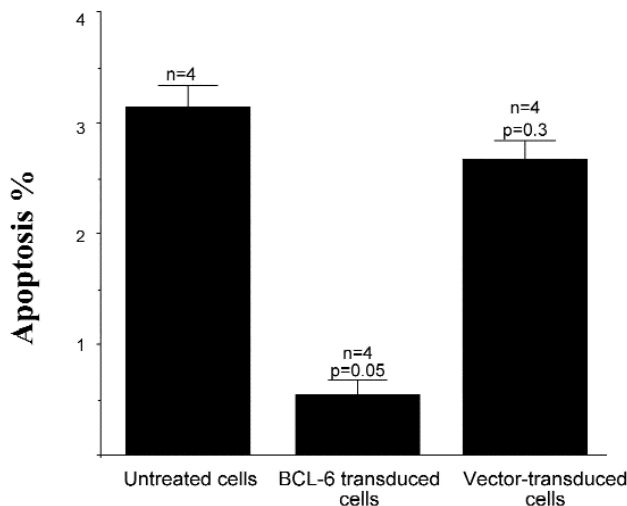


Fig 3: Effects of BCL-6 on growth of Eph4. Cell cycle analysis of Eph4 transduced with BCL-6 or empty retroviral vector alone after selection. The numbers shown the percentage of cells in different phases of the cell cycle. The first peak contains the cells with diploid DNA in G0-G1, the cells in the second peak with twice BrdU-fluorescence intensity are tetraploid in G2-M and the shaded area found between the two peak shows the cells in S-phase. The result shown are representative of four independent experiments with similar results.

G2 are not labeled by BrdUrd and can be discriminated from each other by their different DNA content (PI staining). After 9 hours for Eph4 cell transduced with empty vector and 12 hours for BCL-6 transduced Eph4 cells, BrdUrd-labeled G1 cells begin to appear. These cells were in late S phase at the time of labeling. These results indicate that the cell cycle time of BCL-6-transduced Eph4 cells is prolonged by about 3 hours presumably as a result of the action of BCL-6 at the G1/S transition. They also show that BCL-6 expression does not completely block the proliferation of Eph4 cells, as may have been anticipated from the continued, albeit suppressed, growth of the BCL-6 transduced cells in the liquid cultures. Morphological examination of BCL-6-transduced cells suggested that they were larger than control cells. This impression was confirmed by measurements of cell and nuclear size by the light microscope.

More importantly, using TUNEL as a marker of apoptosis, we found differences in the frequencies of viable and apoptotic cells in cultures of the parent Eph4 cells, control transduced Eph4 cells or BCL-6-transduced Eph4 cells. BCL-6 was able to prevent apoptosis when cells were cultured without serum (Figure 4).

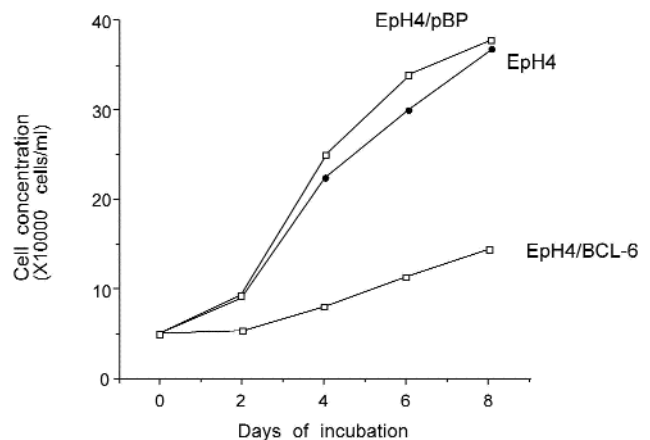


Fig 4: Effects of BCL-6 on apoptosis of Eph4. Number of apoptotic cells assessed by TUNEL assay. Significant difference was found in the frequencies of apoptotic cells in BCL-6-transduced Eph4 cells compared to the parent Eph4 cells and those transduced with vector alone

Discussion

The results show that the transduction procedure per se had no effect on proliferation and confirmed that BCL-6 expression reduces the growth rate. Moreover, the cell cycle results may be attributable to an increased duration of protein synthesis as a result of extended G phase. In contrast, the control cells exhibited G1 cell cycle arrest.

Baron et al have shown that the human programmed cell death-2 (PDCD-2) gene is a target for the BCL-6.⁽¹¹⁾ This finding may support the fact that it inhibits apoptosis. Tang et al¹² showed that BCL-6 is a pro-apoptotic gene. Taken together, it appeared that this transcription factor is associated

with continuing growth and its absence triggers apoptosis. The observed reduction in the growth rate could be a result of an increased level of cell loss by apoptosis. However, we have shown that BCL-6 restoration inhibits the frequency of apoptotic cells compared with the controls. Thus, BCL-6 expression may in fact protect cells from apoptosis which is in agreement with the reduction of the proliferating fraction of BCL-6-restored cells in liquid cultures.

Although it became clear from these results that BCL-6 maintains viability of the mammary epithelium, it is still confusing that those mice with homozygous disruptions of the BCL-6 locus, do not demonstrate any abnormalities of the mammary gland development.

Again this indicates a positive correlation between apoptosis and proliferation. Recent experimental evidence has also suggested that there may be a positive relationship between apoptosis and proliferation. Thus, Traycoff et al¹³ and Reid et al¹⁴ have shown that increased proliferation is linked with an increase in the frequency of apoptotic cells. On the other hand, models proposed by Koury¹⁵ and Alenzi et al¹⁶ imply that a reduction in apoptosis might result in an increase in proliferation, potentially resulting in an inverse relationship between apoptosis and proliferation.¹⁷ Therefore, evidence for a link between apoptosis and proliferation in breast cancer remains unclear.

Since BCL-6 is part of the integrated cell cycle machinery and a key cell cycle regulator, it is now being attributed with a role in regulating proliferation and differentiation. It becomes of interest to explore the link between the control of the cell cycle and cell kinetics. As far as we are aware, this result provides the first evidence of an effect on the outcome of normal cell division of a gene associated with the control of cell cycle parameters. Thus, it creates a link between the control of cell cycle and the control of cell kinetics.¹⁸

Overall, our results propose that BCL-6 maintains cell survival, inhibits apoptosis of mammary epithelium and identifies a novel role for BCL-6 in carcinogenesis. Our data have established an important link between genes that control cell cycle and cell kinetics which may be relevant to oncogenic processes. We have also gained a new insight into the influence of BCL-6 on the growth kinetics of the Eph4 cell line. This contributes to understanding the mechanism whereby the acquisition of BCL-6 may result in malignant transformation and the functions among the key cell cycle regulators that may also be important for the control of proliferation and differentiation.

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References

1. Li S, Rosen JM. Nuclear factor I and mammary gland factor (STAT5) play a critical role in regulating rat whey acidic protein gene expression in transgenic mice. *Mol Cell Biol*. 1995; 15: 2063-70.
2. Huang YC, Hung WC, Kang WY, Chen WT, Chai CY. Expression of STAT3 and Bcl-6 oncoprotein in sodium arsenite-treated SV-40 immortalized human uroepithelial cells. *Toxicol Lett*. 2007;173:57-65.
3. Reljic R, Wagner SD, Peakman LJ, Fearon DT. Suppression of signal transducer and activator of transcription 3-dependent B lymphocyte terminal differentiation by BCL-6. *J Exp Med*. 2000; 192: 1841-8.
4. Xu Y, McKenna RW, Doolittle JE, Hladik CL, Kroft SH. The t(14;18) in diffuse large B-cell lymphoma: correlation with germinal center-associated markers and clinical features. *Appl Immunohistochem Mol Morphol*. 2005;13:116-23.
5. Ye BH, Rao PH, Chaganti RS, Dalla-Favera R. Cloning of BCL-6, the locus involved in chromosome translocations affecting band 3q27 in B-cell lymphoma. *Cancer Res*. 1993; 53: 2732-5.
6. Wagner SD, Kaeda JS. BCL-6: rearrangement and mutation in lymphoma. *Methods Mol Med*. 2005;115:251-70.
7. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science*. 1997; 276: 589-92.
8. Kanazawa N, Moriyama M, Onizuka T, Sugawara K, Mori S. Expression of BCL-6 protein in normal skin and epidermal neoplasms. *Pathol Int*. 1997; 47: 600-7.
9. Lin Z, Kim H, Park H, Kim Y, Cheon J, Kim I. The expression of bcl-2 and bcl-6 protein in normal and malignant transitional epithelium. *Urol Res*. 2003;3:272-5.
10. Bos R, van Diest PJ, van der Groep P, Greijer AE, Hermesen MA, Heijnen I, Meijer GA, Baak JP, Pinedo HM, van der Wall E, Shvarts. A Protein expression of B-cell lymphoma gene 6 (BCL-6) in invasive breast cancer is associated with cyclin D1 and hypoxia-inducible factor-1 alpha (HIF-1 alpha). *Oncogene*. 2003; 22: 8948-51.
11. Baron BW, Anastasi J, Thirman MJ, Furukawa Y, Fears S, Kim DC, Simone F, Birkenbach M, Montag A, Sadhu A, Zeleznik LN, McKeithan TW. The human programmed cell death-2 (PDCD2) gene is a target of BCL6 repression: implications for a role of BCL-6 in the down-regulation of apoptosis. *Proc Natl Acad Sci USA*. 2002; 99: 2860-5.
12. Tang TT, Dowbenko D, Jackson A, Toney L, Lewin DA, Dent AL, Lasky LA. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. *J Biol Chem*. 2002; 277: 14255-65.
13. Traycoff CM, Orazi A, Ladd AC, Rice S, McMahon J, Srour EF. Proliferation-induced decline of primitive hematopoietic progenitor cell activity is coupled with an increase in apoptosis of ex vivo expanded CD34+ cells. *Exp Hematol*. 1998; 26: 53-62.
14. Reid S, Ritchie A, Boring L, Gosling J, Cooper S, Hangoc G, Charo IF, Broxmeyer HE. Enhanced myeloid progenitor cell cycling and apoptosis in mice lacking the chemokine receptor, CCR2. *Blood*. 1999; 93: 1524-33.
15. Koury MJ. Programmed cell death (apoptosis) in hematopoiesis. *Exp Hematol*. 1992; 20: 391-4.
16. Alenzi FQ, Marley SB, Chandrasekaran A, Botto M, Warrens A, Goldman J and Gordon MY. Regulation of haemopoietic progenitor cell number by the Fas/FasL apoptotic mechanism. *Experimental Hematology*. 2002; 30: 1428-35.
17. Alenzi FQ. Links between apoptosis, cell cycle and proliferation. *Br J Biomed Sci*. 2004;61:99-102.
18. Rui L, Goodnow CC. Lymphoma and the control of B cell growth and differentiation. *Curr Mol Med*. 2006;6:291-308.