Mol. Cells, Vol. 12, No. 3, pp. 368-371

Molecules and Cells

The Expression of Bok Is Regulated by Serum in HC11 Mammary Epithelial Cells

Seck-Ho Ha, Sul-Ra Lee, Tae-Hoon Lee[1], Young-Min Kim, Myung-Gi Baik[2], and Yun-Jaie Choi*

Department of Animal Science and Technology, School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea;

[1] Animal Molecular Physiology Research Unit, Korea Research Institute of Bioscience and Biotechnology, Korea Institute of Science and Technology, Daejeon 305-606, Korea;

[2] Department of Genetic Engineering and Institute of Biotechnology, Chonnam National University, Kwangju 500-757, Korea.

(Received July 23, 2001; Accepted September 7, 2001)

Epithelial cells within the mammary gland undergo apoptosis during weaning. To determine the expression of Bok mRNA (a member of the pro-apoptotic Bcl-2 family) in the mammary gland and its regulation, we examined the expression of the Bok transcript in the mouse mammary gland and HC11 mammary epithelial cells in culture through RT-PCR. The Bok mRNA expression was found in the mammary gland. The expression of the Bok mRNA level was induced through serum starvation and overexpression of Bok induced apoptosis in HC11 cells in culture. These results indicate that the expression of Bok mRNA in the mammary gland is regulated through serum starvation. It also may be related to the mammary involution.

Keywords: Apoptosis; Bok; HC11 Mammary Epithelial Cells; Serum Starvation.

Introduction

Programmed cell death (PCD) or apoptosis plays a vital role in normal developmental processes of multicellular eukaryotes, such as embryogenesis, upon elimination of the interdigital web tissue or maturation of the nervous system when supernumerary neurons die (Vaux, 1993; Wyllie, 1995; Wyllie *et al*., 1980). PCD is also important in the elimination process of self-reactive lymphocytes in adults and during the involuting stage of mammary glands after weaning (Choi *et al*., 1996; Cohen, 1993; Schwartz and Osborne, 1993).

Mammary gland development is characterized by proliferative phases during puberty and pregnancy, terminal differentiation, and milk production during lactation. In the involuting mammary glands of mice and several other species, the elimination of most secretory epithelial cells is regulated via apoptosis (Baik *et al*., 1994; Strange *et al*., 1992; Walker *et al*., 1989) and characterized by a decrease in the number of lactogenic hormones. Glucocorticoid prevents involution (Ossowski *et al*., 1979) and PCD of the mammary gland (Feng *et al*., 1995).

Apoptosis involves two essential steps of decision and execution. The Bcl-2 family consists of different anti- and pro-apoptotic members and plays an important role in the decision step. Life or death of a cell is determined based on the ratio between the two members. Bok (Mtd), a pro-apoptotic Bcl-2 protein, induces apoptosis in the brain, liver, lymphoid tissue, ovary, testis, and uterus. It contains BH-1, -2, and -3 domains as well as a C-terminal transmembrane region. Apoptosis from the overexpression of Bok can be blocked by some anti-apoptotic Bcl-2 members (Hsu *et al*., 1997; Inohara *et al*., 1998).

In this study, we revealed the expression of Bok mRNA in a mouse's mammary gland via a reverse transcription-polymerase chain reaction (RT-PCR). In addition, the regulation of the steady-state Bok mRNA level by serum, and the function of Bok in HC11 cells in culture, were also determined.

* To whom correspondence should be addressed.
Tel: 82-31-290-2347; Fax: 82-31-294-1340
E-mail: cyjcow@snu.ac.kr

Abbreviations: G3PDH, glyceraldehyde 3-phosphate dehydrogenase; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide; PCD, programmed cell death; RT-PCR, reverse transcription-polymerase chain reaction.

Materials and Methods

Total RNA isolation and RT-PCR Total RNAs were isolated from a mouse's mammary gland and ovary using the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987), and from HC11 cells with Trizol (GibcoBRL, USA), following the manufacturer's protocol. First-strand cDNAs were synthesized from 1 μg of total RNA in a 10 μl reaction volume using an oligo dT primer and a SMART™ PCR cDNA synthesis kit (Clontech, USA). Bok cDNA was amplified in a 50 μl reaction mixture that contained RT-products (2 μl), 1× PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTP, 1.25U Taq DNA polymerase, and 10 pmol each of sense- and anti-sense primers that were derived from the mouse Bok cDNA sequence published by Inohara *et al.* (1998). The sense primer (5′-CCACATCTTCTCAG-CAGGTATC-3′) represents the 22 nucleotides of the mouse cDNA coding sequence. The anti-sense primer (5′-AAGAGC-TAGAGCAGT-CACAGAG-3′) was complementary to the 22 nucleotides of the noncoding sequence. G3PDH cDNA was amplified with the primers purchased from Clontech (USA) and was used to control the amount of RNA in RT-PCR. For the amplification of Bok cDNA from the mammary gland and HC11 samples, PCR conditions were set at 30 cycles of denaturation at 95°C for 15 s, annealing at 61°C for 20 s, and extension at 72°C for 1 min.

Cell culture and transfection HC11 cells were cultured in a growth medium that contained RPMI1640 (GibcoBRL), 50 μg/ml gentamycin (Sigma, USA), 10% fetal bovine serum (FBS, GibcoBRL), 5 μg/ml insulin (Sigma), and 10 ng/ml EGF (Sigma) (Lee *et al.*, 1997). The cells were transfected using Lipofectamine Plus ™Reagent (GibcoBRL) with pcDNA3-Bok (sense), pcDNA3-αBok (antisense), or pcDNA3 only as a control, together with a 1/10 fraction of a transfected cell indicator. They were incubated with plasmids in a serum-free medium for 4 h, followed by the addition of FBS to a final concentration of 5%, then further incubated for 14 h. After additional culture in a fresh medium for 16 h, the cells were fixed in 0.25% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (0.4 mg/ml) for the detection of β-gal expression.

Viability assay HC11 cells were seeded on 96-well plates at a density of 20,000 cells/well. On the following day, the cells were serum-starved for 24 h, and viable cell numbers were estimated using a Cell Proliferation Kit I (MTT) (Roche, USA) according to the manufacturer's procedure. Absorbance at 620 nm was subtracted from that at 540 nm for each well.

Results and Discussion

Current theories for the regulation of apoptosis by Bcl-2 family members rely heavily on the ratio of death promoter to death inhibitor (Boyd *et al.*, 1995; Oltvai *et al.*, 1993; White, 1996). Bok is a pro-apoptotic Bcl-2 family member (Hsu *et al.*, 1997). Its transcripts of 1.5 kb are abundant in the ovary, testis, and uterus, while negligible in other tissues when examined through Northern blot analysis (Hsu *et al.*, 1997). However, the Bok expression has also been reported in other tissues such as the brain, liver, and lymphoid tissues (Inohara *et al.*, 1998).

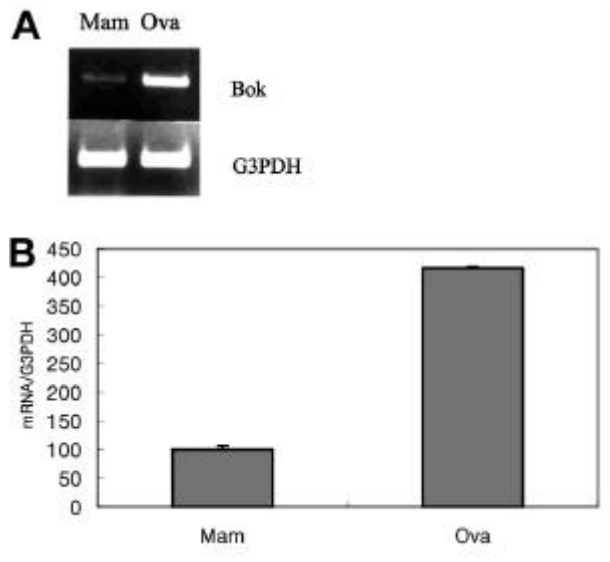

Fig. 1. Expression of Bok in mouse's mammary gland and ovary. **A.** cDNA from the mammary glands (Mam) and ovary (Ova) was amplified with Bok (upper) and G3PDH (down) primers. **B.** Relative levels of Bok mRNA were normalized with corresponding G3PDH levels. mRNA levels at involution-1 day were expressed as 100%. The bar indicates standard deviation of the three independent experiments.

To determine the Bok mRNA expression in mammary gland tissue, we examined the level of Bok mRNA in a mouse's mammary gland through RT-PCR. The expression level was compared with that of the ovary tissue. A 518 bp band was detected in the mouse mammary gland and ovary tissues (Fig. 1A). The detected band had the right size according to the design of the primers used in PCR. The sequence analysis also showed that the PCR product was identical to the Bok nucleotide. The mRNA level of the mammary gland was lower than that of the ovary (Fig. 1B). This result suggests that Bok may be related to the control of mammary gland development and apoptosis.

Possible regulation of apoptosis by Bok in HC11 mammary epithelial cells that were derived from the BALB/C mouse mammary epithelial cell line (Ball *et al.*, 1988) was examined. This cell line was chosen for the mammary gland study due to its uniqueness in maintaining the capability to produce the major mouse milk protein (β-casein) under the control of lactogenic hormones, such as insulin, dexamethasone, and prolactin. In addition, many investigators have successfully used this cell line to study

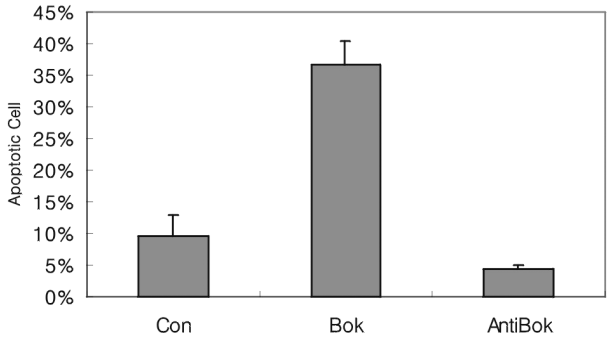

Fig. 2. Bok-induced apoptosis in HC11 mammary epithelial cells. 3×10^5 HC11 cells were co-transfected with pcDNA3 (Con), pcDNA3-Bok (Bok), or pcDNA3-αBok (AntiBok) together with pCMVβ-gal. After transfection, the cells were stained with β-galactosidase and examined for morphological signs of apoptosis. The graph shows the percentages of round blue apoptotic cells (mean ± SD).

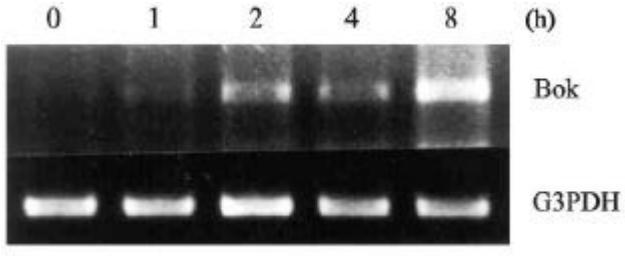

Fig. 3. Expression of Bok after serum starvation in HC11 cells. HC11 cells were serum-starved for 0, 1, 2, 4, and 8 h, and the total RNA was isolated. Amplification and three repeats were performed, as described in Fig. 1.

the regulation of mammary involution specific gene expression.

Expression vectors that contain Bok in either sense or antisense orientation were constructed and transfected to the HC11 cells. The transfection of the HC11 cells with Bok, but not with the antisense construct, significantly increased the number of apoptotic cells. This suggests that Bok also induces apoptosis in mammary epithelial cells (Fig. 2).

Serum starvation induces apoptosis in HC11 cells in high cell density cultures (Lee *et al.*, 1999; Merio *et al.*, 1997). HC11 cells were subjected to serum starvation using a medium that contained only RPMI1640 in a time course. The level of Bok mRNA in the cells was measured at each time point. While the Bok expression gradually increased after serum starvation, the level of G3PDH mRNA did not change (Fig. 3). At the same time, we ex-amined the expression of the Bcl-2 gene, and the result showed that there was no indication of change in the Bcl-2 level (data not shown). The effect of serum on the ex-

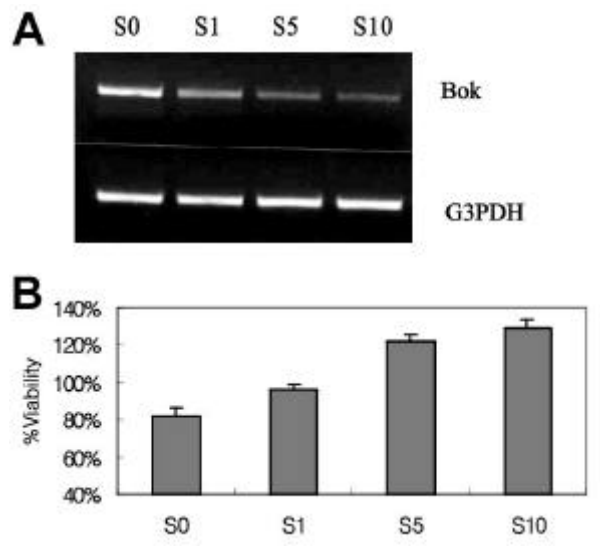

Fig. 4. Expression of Bok and cell viability on various serum concentrations. HC11 cells were incubated for 24 h in a medium that contained 0 (S0), 1 (S1), 5 (S5), and 10% (S10) serum. **A.** Total RNA was prepared from each treatment. Amplification and three repeats were performed as described in Fig. 1. **B.** Viability was assessed through the MTT assay. The values are shown as mean ± SD of at least three independent experiments.

pression of the Bok gene was confirmed using various concentrations of serum from 0 to 10%. With the increase in serum concentration, the mRNA level of Bok decreased, while the cell viability increased, after 24 h of treatment (Fig. 4).

Many pro- and anti-apoptotic members are activated through post-translational modification and/or conformational changes. Although some pro-apoptotic members are transcriptionally silent, cells initiate their transcription in response to selected death stimuli. Hrk, which is predicted to be a constitutively active pro-apoptotic member, can be up-regulated in response to the death stimuli (Inohara *et al.*, 1997). Indeed, Bax appears to be transcriptionally responsive to p53 induction (Miyashita and Reed, 1995). Our results show that the expression of Bok was regulated by serum and induced apoptosis in mammary epithelial cells. Therefore, we demonstrated that pro-apoptotic member Bok may be transcriptionally regulated by serum. It may play a role in the mammary involution.

Acknowledgments This work was supported by a grant from the Korea Research Foundation (1998-019-G00024).

References

Baik, M. G., Seol, J. K., and Choi, Y. J. (1995) The induction of Fas mRNA and DNA fragmentation in the involution stage of mouse mammary gland. *Mol. Cells* **5**, 81-85.

Ball, R. K., Friis, R. R., Schoenenberger, C. A., Doppler, W., and Grouer, B. (1988) Prolactin regulation of β-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. *EMBO J.* **7**, 2089-2095.

Boyd, J. M., Gallo, G. J., Elangovan, B., Houghton, A. B., Malstrom, S., and Awery, B. J. (1995) Bik1, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival promoting proteins. *Oncogene* **11**, 1921-1927.

Choi, B. H., Myung, K. H., Lee, M. J., Kim, H. H., Jeon, D. H., Hwang, I. T., Choi, Y. J., Paik, S. G., and Baik, M. G. (1996) Cloning of involution-induced cDNAs from mouse mammary gland. *Mol. Cells* **6**, 311-315.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiothyanate-phenol-chloroform extraction. *Anal. Biochem*. **161**, 156-159.

Cohen, J. J. (1993) Apoptosis. *Immunol. Today* **14**, 126-130.

Feng, Z., Marti, A., Jehn, B., Altermatt, H. J., Chicaiza, G., and Jaggi, R. (1995) Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. *J. Cell Biol*. **131**, 1095-1103.

Hsu, S. Y., Kaipia, A., Mcgee, E., Lomell, M., and Hsueh, A. J. W. (1997) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc. Natl. Acad. Sci. USA* **94**, 12401-12406.

Inohara, N., Ding, L., Chen, S., and Nunez, G. (1997) Harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival promoting proteins Bcl-2 and Bcl-X(L). *EMBO J.* **16**, 1686-1694.

Inohara, N., Ekhterae, D., Garcia, I., Carrio, R., Merino, J., Merry, A., Chen, S., and Nunez, G. (1998) Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-XL. *J. Biol. Chem.* **273**, 8705-8710.

Lee, D. Y., Ha, S. H., Kho, Y. J., Kim, J. Y., Cho, K. K., Baik, M. G., and Choi, Y. J. (1999) Induction of mouse Ca^{2+}-sensitive chloride channel2 gene during involution of mammary gland. *Biochem. Biophys. Res. Commun.* **264**, 933-937.

Lee, M. J., Hwang, I. T., Choi, Y. J., Paik, S. G., Lee, H. B., and Baik, M. G. (1997) EGF inhibits expression of WDNM1 and sulfated glycoprotein-2 genes in mammary epithelial cells. *Biochem. Biophys. Res. Commun.* **240**, 104-107.

Merlo, G. R., Basolo, F., Fiore, L., Duboc, L., and Hynes, N. E. (1995) p53-dependent and p53-independent activation of apoptosis in mammary epithelial cells reveals a survival function of EGF and insulin. *J. Cell Biol*. **128**, 1185-1196.

Miyashita, T. and Reed, J. C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**, 293-299.

Oltvai, Z. N., Millman, C. L., and Korsmeyer, S. J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619.

Ossowski, L., Biegel, D., and Reich, E. (1979) Mammary plasminogen activator correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. *Cell* **16**, 929-940.

Schwartz, L. M. and Osborne, B. A. (1993) Programmed cell death, apoptosis and killer genes. *Immunol. Today* **14**, 582-590.

Strange, R., Li, F., Friis, R. R., Reichmann, E., Haenni, B., and Burri, P. H. (1992) Apoptotic cell death and tissue remodeling during mouse mammary gland involution. *Development* **115**, 49-58.

Vaux, D. L. (1993) Towards an understanding of the molecular mechanism of physiological cell death. *Proc. Natl. Acad. Sci. USA* **90**, 786-789.

Walker, N. I., Bennett, R. E., and Kerr, J. F. R. (1989) Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am. J. Anat*. **185**, 19-32.

White, E. (1996) Life, death and the pursuit of apoptosis. *Genes Dev.* **10**, 1-15.

Wyllie, A. H. (1995) The genetic regulation of apoptosis. *Curr. Opin. Genet. Dev.* **5**, 97-104.

Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) Cell death the significance of apoptosis. *Int. Rev. Cytol*. **68**, 251-258.