INTRODUCTION

After the lactation period, the mammary gland undergoes an extensive remodeling process that leads to the involution of epithelial structures (Walker et al., 1989; Strange et al., 1992). The involution phase of mammary gland development is characterized by epithelial cell death. The involution process occurs in two phases. During the first phase, accumulation of milk is associated with an engorgement of the gland, with a marked change in the pattern of gene expression and with massive apoptosis of epithelial cells (Walker et al., 1989; Strange et al., 1992; Marti et al., 1999). Accumulation of factors in the milk, the shape change of epithelial cells due to the engorgement, changes of hormone levels and loss of survival factor function are possible triggers of this initial phase of apoptosis (Topper and Freeman, 1980; Feng et al., 1995; Li et al., 1997; Chapman et al., 1999; Marti et al., 1997, 1999).

However, it remains still unclear how these changes are translated into an apoptotic response in milk producing mammary epithelial cells. During the second phase, extracellular matrix degrading proteases are produced that may be responsible for the collapse of lobulo-alveolar structures and the subsequent tissue remodeling (Talhouk et al., 1992; Lund et al., 1996; Li et al., 1997).

The induction of several genes including stromelysin, Fas antigen, Bok, interleukin-10, lysozyme, and interleukin-1β converting enzyme has been reported during involution of the mammary gland and in apoptotic mammary epithelial HC11 cells (Boudreau et al., 1995; Ha et al., 2001; Lee et al., 2001; Kim et al., 2003). In previous study, we observed induction of extracellular protease inhibitor (Expi) gene in apoptotic mammary epithelial cells (Jung et al., 2004). The Expi, previously known as WDNM1 (Dear et al., 1988, 1989), is a member of the four-disulfide core family of proteins that share a characteristic pattern of cysteine residues...
forming intrachain disulfide bonds involved in stabilizing protein structure. Our previous results show that the expression of Expi gene was induced during involution of mammary gland (Jung et al., 2004). Overexpression of Expi gene accelerated apoptosis of mammary epithelial cells, and induced expression of B cell activating factor (BAFF).

BAFF is a recently identified member of the tumor necrosis factor (TNF) family of ligand. A proliferation-inducing ligand (APRIL), another TNF family member, is the closest known relative of BAFF and is produced by macrophages (Hahne et al., 1998). APRIL is involved in the regulation of death ligand-induced apoptotic signaling in malignant glioma cells (Roth et al., 2001). Three BAFF receptors, B-cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI) and BAFF-receptor (BAFF-R), have been known (Laabi et al., 2001). BAFF preferentially binds to BAFF-R and TACI and interacts with BCMA more weakly. Previously, only expression of the BAFF-R was detected and induced in the Expi-transfected cells, while the TACI and the BCMA expression was not detected in mammary epithelial cells (Jung et al., 2004). BAFF and APRIL also share two receptors, TACI and BCMA. But, APRIL binds more avidly to BCMA than to TACI and does not bind to BAFF-R. It has been suggested that APRIL receptor exists and expects to be expressed on epithelial cells. BAFF and APRIL pathways and expression patterns of related genes have not been studies in mammary epithelial cells.

In other cell types, NF-κB and bcl-2 family genes have been postulated to involve in BAFF and APRIL pathways. There is no report on BAFF and APRIL expression in the involution stage of mammary gland at which active apoptosis occurs in mammary epithelial cells. Purpose of this study was to examine expression patterns of BAFF and APRIL pathway-related genes including BAFF-R, NF-κB and bcl-2 family genes in the Expi-transfected HC11 cells and in various reproductive stages of mouse mammary gland.

### MATERIALS AND METHODS

**Culture of Expi-transfected mammary epithelial HC11 cells**

In this study, previously developed cell lines overexpressing Expi gene were used (Jung et al., 2004). Briefly, Expi gene expression vector was constructed by ligating into pBK-CMV vector, and the recombinant DNA was transfected into HC11 cells using lipofectamine method. After G418 selection, we isolated colonies of pExpi- and of pNeo-transfected cells. HC11 cells were cultured in RPMI1640 growth medium (Gibco BRL, USA) containing 10% fetal bovine serum (Gibco BRL), 5 × 10⁻⁶M b-mercaptoethanol, and blotted onto a membrane. cDNAs of BAFF, BAFF-R, APRIL, NF-κB, Bcl-2, Bcl-xL and Bax genes were amplified by RT (reverse transcriptase)-PCR using total RNA templates isolated from mammary gland. Mouse mammary tissues were also prepared from pregnancy, lactation and involution stages for northern analysis. Mouse mammary tissues were also prepared from pregnant day 12, lactating day 6, and involution days 1 and 2 for one experiment. For the induction of involution, the young were removed 10 days after parturition, and the mammary tissues were obtained at the indicated time after weaning.

Total RNA was extracted by the acid guanidinium thiocyanate phenol/chloroform method (Chomczynski and Sacchi, 1987). Twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane. cDNAs of BAFF, BAFF-R, APRIL, NF-κB, Bcl-2, Bcl-xL and Bax genes were amplified by RT (reverse transcriptase)-PCR using total RNA templates isolated from mammary gland. Primer information was shown in Table 1. PCR reaction was performed using Taq polymerase for 30 cycles at 55°C annealing temp. RT-PCR products were purified using AccuPrep™ PCR purification kit (Bioneer, Korea). Purified PCR products were cloned into TA cloning vector, pCR2.1

### Table 1. Primer information for RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>5'-primer</th>
<th>3'-primer</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAFF</td>
<td>gagaatggatgagtgcacagcc</td>
<td>atcagcgaagttgcacagcc</td>
<td>948</td>
<td>Schneider et al., 1999</td>
</tr>
<tr>
<td>BAFF-R</td>
<td>acgtcagctacgtacagcc</td>
<td>acgtcagctacgtacagcc</td>
<td>540</td>
<td>Thompson et al., 2001</td>
</tr>
<tr>
<td>APRIL</td>
<td>ggtccctagctgatcagcc</td>
<td>ggtccatgcggagaagcc</td>
<td>710</td>
<td>Yu et al., 2000</td>
</tr>
<tr>
<td>NF-κB</td>
<td>ggtccctagctgatcagcc</td>
<td>ggtccatgcggagaagcc</td>
<td>450</td>
<td>Oltvai et al., 1993</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>ggtccctagctgatcagcc</td>
<td>ggtccatgcggagaagcc</td>
<td>580</td>
<td>Negrini et al., 1987</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>ggtccctagctgatcagcc</td>
<td>ggtccatgcggagaagcc</td>
<td>557</td>
<td>Gonzalez-Garcia et al., 1994</td>
</tr>
</tbody>
</table>

**Tissue sampling and northern analysis**

The ICR mouse mammary membrane (Seegene, Korea), which is pre-made for immediate use, was analyzed for northern analyses of BAFF, BAFF-R, APRIL and NF-κB genes. Mouse mammary tissues were also prepared from pregnancy, lactation and involution stages for northern analysis of bcl-2, bcl-xL and bax genes. Since mice have relatively small amounts of tissues at involution 3, 4, and 7 days, mammary tissues were collected and pooled from four animals at each stage, and used for a northern analysis. Mammary tissues were pooled from three animals at pregnant day 12, lactating day 6, and involution days 1 and 2 for one experiment. For the induction of involution, the young were removed 10 days after parturition, and the mammary tissues were obtained at the indicated time after weaning.

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(Invitrogen, USA), and correct nucleotide information was confirmed by nucleotide sequencing. The plasmid was digested with EcoR I, and the insert was obtained after low melting agarose gel electrophoresis. The insert of cDNA clone was labeled using a Prime-It Random Primer Labeling Kit (Stratagene). The membrane was hybridized with the 32P-labeled insert of the cDNA clone. The equal amount of RNA loading was confirmed by the intensities of 28S and 18S bands, and the efficiency of transfer was monitored by ethidium bromide staining.

The membrane was prehybridized with the prehybridization solution (10% dextran sulfate, 0.5% SDS, 6×SSC, 1 mM EDTA, 100 µg/ml salmon sperm DNA) at 65°C for 1 h, and the cDNA probe preheated at 95°C was added, and hybridization was performed at 65°C for 20 h. The membranes were washed twice in 2×SSC/0.1% SDS at room temperature for 10 min, once in 2×SSC/0.1% SDS at 42°C for 30 min, once in 0.1×SSC/0.1% SDS at 42°C for 30 min, and once in 0.1×SSC/0.1% SDS at 55°C for 30 min, and once in 0.1×SSC/0.1% SDS at 68°C for 30 min. The membranes were exposed to phosphoimage cassette at room temperature for 24-48 h.

**RESULTS AND DISCUSSION**

Expression of BAFF-R, APRIL and NF-κB genes in Expi-transfected mammary epithelial cells

The Expi is a member of the four-disulfide core family of proteins (Dear and Kefford, 1991). Previously, we found that the overexpression of Expi accelerated the apoptosis of mammary epithelial cells under serum starvation (Jung et al., 2004), and induction of B cell activating factor (BAFF) gene expression was observed in the Expi-overexpressed apoptotic cells. BAFF is a survival/maturation factor for peripheral B cells and this activity is mediated through a BAFF-specific receptor, BAFF-R. BAFF and APRIL are two related members of the TNF ligand superfamily (Mackay and Ambrose, 2003). APRIL plays a role in T-independent type II antigen responses and T cell survival, but can also induce proliferation/survival of non-lymphoid cells. In the current study, expression levels of BAFF and APRIL pathway-related genes were examined by northern analysis in pExpi-transfected cells at 70% confluent stage and 0 h and 72 h incubation in serum-free media. Expi-transfection showed a strong upregulation (3-5 folds) of BAFF-R gene expression at 70% confluent stage and at 0 h and 72 h incubation in serum-free media. Expi-transfection showed a strong upregulation (3-5 folds) of BAFF-R gene expression at 70% confluent stage and at 0 h and 72 h incubation in serum-free media. Expi-transfection showed a strong upregulation (3-5 folds) of BAFF-R gene expression at 70% confluent stage and at 0 h and 72 h incubation in serum-free media.

![Figure 1](image-url)
Expression of BAFF pathway and Bcl-2 family genes in mouse mammary gland

BAFF signals were mediated via BAFF-R, NF-κB and Bcl-2 family genes in B cells. BAFF signaling pathways have not been studied in mammary epithelial cells. Expression pattern of BAFF and APRIL pathway-related genes was examined in mouse mammary gland at various physiological stages including virgin, pregnancy (5.5, 8.5, 13.5 and 18.5 days), lactation (2 and 8 days), and involution stages (1, 2, 4, and 7 days). Expression levels of BAFF gene were very low at early pregnancy (5.5 and 8.5 days of pregnancy), increased from mid-pregnancy (13.5 days of pregnancy), and peaked at lactation, and expression levels were slightly decreased at involution stages of mammary gland (Figure 2). Thus, BAFF transcripts seem to be abundant from late pregnancy through lactation and involution stages, and expression patterns of BAFF gene are not correlated to apoptotic status of mammary gland: active apoptosis of mammary epithelial cells occurs at involution stage of mammary gland. Expression levels of BAFF-receptor (BAFF-R) gene were relatively high at virgin through mid pregnancy, the expression was decreased at lactation stages, then the expression was highly induced from involution day 2 and high levels were maintained until involution days 7. Results suggest that expression patterns of BAFF-R are more correlated with apoptosis of mammary epithelial cells than BAFF molecules itself. Expression levels of APRIL gene were low at virgin and early pregnancy, increased at mid- and late-pregnancy, and then decreased during lactation and slightly increased during involution. Further study is required to understand functional role of BAFF pathway in mammary gland.

The NF-κB pathways were used by the receptors for BAFF and APRIL in B cells. BAFF-induced BAFF-R signaling is primarily mediated via the alternative NF-κB pathway. This pathway leads to cleavage of p100 to p52 (NF-κB-2). A dimer of p52 and RelB is translocated into the nucleus where it activates target gene transcription. BAFF may also activate the classical NF-κB pathway. We also examined expression of NF-κB gene in mammary gland. Expression patterns of NF-κB gene were similar as those of BAFF-R: high levels were maintained from virgin through mid-pregnancy, the levels decreased sharply at lactation and increased from involution day 2, and high levels were maintained until involution days 7.

The NF-κB family of transcription factors has been implicated in such diverse cellular processes as proliferation, differentiation, and apoptosis. Generally, the NF-κB activation delivers a survival signal (Beg and Baltimore, 1996; Van Antwerp et al., 1996) by promoting the expression of survival factors, such as some members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, and XIAP) (Rayet and Gelinas, 1999) and bcl-xL (Visconti,
In contrast, current study shows induction of NF-κB gene expression during involution of mammary gland, at which stage active cell death occurs in mammary epithelial cells. Clarkson et al. (2000) also described the activation of NF-κB during involution of the mouse mammary gland. But, active NF-κB localized exclusively to nonapoptotic epithelial cells both in vivo and in the mammary epithelial cell line, KIM-2 (Clarkson et al., 2000). They have suggested that NF-κB might have a selective survival function in epithelial cells.

There are also reports about a pro-apoptotic role for NF-κB (Kim et al., 2006). Several NF-κB stimulators such as TNF (Barger et al., 1995), ceramide (Hunot et al., 1997), H2O2, and serum deprivation (Grimm et al., 1996) ultimately induce apoptosis. The NF-κB-induced death receptor 4 (DR4), DR5 (Ravi et al., 2001), TNF-related apoptosis-inducing ligand (TRAIL) (Rivera-Walsh et al., 2001), Fas (Zheng et al., 2001), and Fas ligand (FasL) (Lin et al., 1999) all may promote cell death. The upstream promoter regions of several death genes contain potential NF-κB binding motifs (Grimm et al., 1996). These conflicting studies of the role of NF-κB in apoptosis suggest that the effects of many signaling molecules are dependent upon cell content and/or stress.

We analyzed mRNA levels of bcl-2 family member bcl-2, bcl-xL and bax genes. Pro-apoptotic bax mRNA levels were upregulated at the onset of involution, with a highest increase in expression occurring at the lactation to involution transition (Figure 3). Previous studies also showed induction of bax gene expression during involution of mouse mammary gland (Metcalfe et al., 1999; Walton et al., 2001). Expression of death-suppressors bcl-2 and bcl-xL was examined in mammary gland. Bcl-2 expression was downregulated during lactation and involution compared to pregnancy, and bcl-2 levels were constant during lactation and involution. Previously, transcripts of bcl-2 gene were not detected at any developmental stages of mammary gland (Walton et al., 2001). Other study showed that expression of bcl-2 gene was low and similar during lactation and early involution, while expression increased during late involution (Schorr et al., 1999). Bcl-xL levels were decreased during lactation compared to pregnancy, and there was a slight increase in bcl-xL levels during involution compared to lactation.

In summary, our results suggest that signaling pathways activated by both BAFF and ARRIL in mammary gland point towards NF-κB activation which causes upregulation of bax. A further study is needed to test whether BAFF and NF-κB molecules have significant roles in either proliferation or apoptosis of mammary epithelial cells during involution of mammary gland.

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REFERENCES


