NF-κB Inhibits Apoptosis in Murine Mammary Epithelia*

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The transcription factor NF-κB is a key modulator of apoptosis in a variety of cell types, but to date this specific function of NF-κB has not been demonstrated in epithelia. Here, we describe the activation of NF-κB during post-lactational involution of the mouse mammary gland, a period of extensive apoptosis of luminal epithelial cells. Significantly, active NF-κB localized exclusively to nonapoptotic epithelial cells both in vivo and in the mammary epithelial cell line, KIM-2, transfected with an NF-κB-dependent green fluorescent protein reporter. Activation of NF-κB in vitro coincided with a decrease in the cytosolic repressor, IkBα. Furthermore, induction of NF-κB either by extracellular ligands or, more specifically, by inhibition of the IkB repressor with adenoviral constructs expressing antisense mRNA, resulted in enhanced survival of KIM-2 cells. Therefore, although coincident with induction of apoptosis both in vivo and in vitro, NF-κB appeared to exert a selective survival function in epithelial cells. This study highlights for the first time a role for NF-κB in modulating apoptosis in epithelium.

Tissue homeostasis during development requires an appropriate balance of pro-apoptotic and survival signals from the extracellular environment. The intracellular targets of these signals are well known and include genes encoding cytosolic effectors and specific inhibitors of the terminal apoptotic cascade (1, 2). Much less is known about the transcriptional regulators responsible for the appropriate expression of these apoptosis-related genes, although it is probable that these genetic mechanisms are critical to the modulation of apoptosis in normal development, differentiation, and tissue modeling. The importance of this level of regulation is highlighted by the range of clinical abnormalities resulting from disregulated apoptosis, including cancer, autoimmune and neurodegenerative disorders, myocardial infarction, stroke, and liver disease (3). Unraveling the genetic mechanisms responsible for controlling susceptibility to apoptosis is essential for understanding both these abnormal states and many aspects of normal development.

The transcription factor NF-κB1 is one of a growing list of transcriptional regulators known to directly affect apoptosis in a tissue specific manner (4, 5). Thus, NF-κB has been shown to affect apoptosis in liver, embryonic fibroblasts, endothelial cells, and neurones (6–12). However, there is little direct evidence hitherto that NF-κB mediates survival or death in epithelia (13, 14). Clearly, a role for NF-κB in epithelial apoptosis could have significant implications for understanding the pathogenesis of a number of diseases.

In an attempt to identify whether NF-κB directly regulates apoptosis in epithelium, we have studied the activity of this transcription factor during post-lactational involution, one of the most dramatic examples of a developmentally regulated epithelial apoptosis in mammals. Involution is characterized by an increase in the number of apoptotic events in the luminal epithelial layer of the lobulo-alveolar compartment soon after weaning (15, 16). This is followed by proteolytic degradation of supporting basement membrane and major remodeling of the gland to a near pre-pregnant morphology (15, 16). Maintenance of the differentiated structure of the gland and survival of the secretory epithelium during lactation depends upon a combination of extracellular survival signals originating from neighboring basement membrane, cell adhesion molecules, lactogenic hormones, and as yet undefined local factors (16–20). Disruption of these signals is known to potentiate apoptosis of mammary epithelial cells, but the downstream effectors of these pro-apoptotic signals remain elusive.

A number of transcription factors are regulated during the transition from lactation to involution in mammary gland, including AP-1, p53, Stas, Ets isoforms, c/ebpα, NF-1, and c-Myc (15, 17, 20–24). Few of these have yet to be shown to directly influence apoptosis in the mammary gland, although we have recently demonstrated delayed involution in conditional Stat3 null mice (23) and NF-κB activation during involution (25). In addition, three studies have demonstrated that specific NF-κB subunits are elevated in breast tumors and transformed mammary epithelial cell lines (26–28). Thus, although no direct effect on apoptosis has been demonstrated to date, the implication of these observations is that inappropriate expression of NF-κB may contribute to mammary epithelial carcinogenesis through aberrant regulation of apoptosis in the developing or regressing mammary gland.

In this study, we address the role of NF-κB activation on survival of normal mammary epithelial cells. First, we describe the activation and epithelial localization of NF-κB in mammary gland during involution. Then, by specifically altering NF-κB activity in a conditionally immortal cell line, KIM-2, we demonstrate that NF-κB suppresses physiological apoptosis in kin 1α; TNF-α, tumor necrosis factor-α; GFP, green fluorescent protein; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; HIV, human immunodeficiency virus.

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1 The abbreviations used are: NF-κB, nuclear factor κB; DM, differentiation medium; AM, apoptosis induction medium; DMEM, Dulbecco’s modified Eagle’s medium; LPS, lipopolysaccharide; IL-1α, interleukin 1α; TNF-α, tumor necrosis factor-α; GFP, green fluorescent protein; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; HIV, human immunodeficiency virus.

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NF-κB Inhibits Death in Mammary Epithelia

mammary epithelial cells. In the light of these observations, we suggest that NF-κB modulates survival of luminal epithelium, specifically during the process of involution; this is the first direct evidence that NF-κB negatively regulates apoptosis in epithelial cells.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents were obtained from Life Technologies, Inc. Popo-1 and streptavidin-Texas Red conjugate were from Molecular Probes (Leiden, Netherlands). Fluorescein-conjugated and biotinylated annexin V were from R & D Systems (OXON, UK). All other reagents were obtained from Sigma. Affinity-purified polyclonal antibodies to p65 (A) and p50 (C-19) were from Santa Cruz Biotechnology, and monoclonal antibodies to p52 and IxBα (10B) were derived as described previously (29).

Reporter and Expression Vectors—The NF-κB promoter reporter vector, pEGFP-HIV-LTR, was constructed by inserting a 739-base pair BamHI/HindIII fragment from the vector pLTRXLUC (30) into the BamHI/HindIII sites of the enhanced GFP reporter vector pEGFP-1 (CLONTECH, Hampshire, UK). The adenosine antisense vector adV-askB was constructed by insertion of the cDNA for IxBα into the EcoRV site of pAd.CMV-link (obtained from P. Moullier, Nantes, France) in the antisense orientation with respect to the cytomegalovirus (CMV) promoter. Adenovirus type 5 cleaved with ClaI was co-transfected into 293 cells with the Nhel linearized plasmid containing the IxBα cDNA and recombinants isolated by 3 rounds of plaque purification. This virus and an adenosine expressing β-galactosidase (adV-LacZ) were propagated in 293 cells. Virus was purified on CsCl gradients as described (31), dialysed against culture medium, and stored at −70 °C.

Cell Culture—KIM-2 cells were maintained as described previously (32). Cells were grown to confluence and transferred to differentiation medium (DM; Ham’s F12/DMEM, 10% fetal calf serum, 1 mg/ml insulin, 40 ng/ml dexamethasone, 5 mg/ml linoleic acid) at a titer of 100 plaque-forming units/cell. After 1 h, 3 ml of DM was added, and cells were incubated for a further 4 h. At this time, DM or AM medium was applied, and cells were incubated for 17 h prior to harvesting.

Immunohistochemistry—Immunohistochemistry for NF-κB p65 was carried out using a rabbit polyclonal antibody to p65 and the peroxidase-based Envision system (Dako Ltd., Cambridge, UK) as described previously (23).

Annexin V Assay—For flow cytometry, KIM-2 cells were washed in PBS/1 mM EDTA, incubated for 10 min in PBS/0.25% trypsin/1 mM EGTA at 37 °C, and transferred to DMEM/10% fetal calf serum. Cell pellets were resuspended in PBS/1% serum and 1 × 10⁶ cells were resuspended in 100 μl of annexin binding buffer (R & D Systems), 5 μg/ml propidium iodide, and 1 μg/ml fluorescein isothiocyanate-annexin V for 15 min in the dark prior to counting. For fluorescence microscopy, cells grown on Permanox slide flasks (Life Technologies) were washed in PBS and incubated in the dark for 15 min with 100 μl of annexin binding buffer, 2 μg popo-1, and either 1 μg/ml fluorescein isothiocyanate-annexin V or 0.5 μg/ml Biotin-annexin V. For Biotin-annexin V, slides were washed in 100 μl of annexin binding buffer and incubated in the dark with 1/2000 Texas Red-conjugated streptavidin.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts from tissue culture cells (33) and mammary gland tissue (34) were performed as described previously. 10 μg of nuclear protein was incubated with a 32P end-labeled oligonucleotide probe containing the NF-κB binding site motifs from the HIV type 1 enhancer, electrophoresed on a 7% native polyacrylamide gel, dried, and autoradiographed. For supershift assays, 0.5 μg of antibody was co-incubated with binding reaction buffer for 2 h on ice.

Western blot Analysis—Cytoplasmic extracts (16 μg) were electrophoresed on a 10% denaturing polyacrylamide gel and electroblotted onto Hybond-C membrane (Amersham Pharmacia Biotech). Membranes were blocked overnight in PBS, 0.1% Tween 20, 5% Marvel and incubated for 1 h with antibody. Following a further 1-h incubation with secondary antibody, bound antibody was detected by ECL reagent. Subsequently, membranes were stripped and incubated with polyclonal antibody p65(A) and detected as above.

RESULTS

NF-κB Activity during Mammary Development—Endogenous NF-κB activity was identified by EMSA in murine mammary tissue during normal development. NF-κB exhibited stage specific changes in DNA binding activity throughout the developmental cycle (Fig. 1A). A high level of activity during gestation suggested a role for NF-κB early in adult mammary development. NF-κB activity then became undetectable during lactation but was evident 1 h after pup withdrawal and remained at low levels for the first 8 h before subsequently increasing to a maximum at 3 days of involution. This rise in NF-κB activity coincided with an increased number of apoptotic cells in the mammary gland and a concomitant decrease in the systemic levels of lacticogenic hormones (15, 20, 35, 36). Similarly, an increase in mobility of the DNA-protein complexes, first evident after 3 days of involution, correlated with an increase in metalloproteinase activity and the onset of major remodeling of the gland. Two predominant species were observed in gel shift assays, both exhibiting similar changes in intensity throughout the time course. The more slowly migrating species consisted almost exclusively of p65 and p50 subunits, whereas the faster migrating species consisted of p50 but little or no p65 (Fig. 1B). Additional isoforms other than p52 (Fig. 1B) may be responsible for residual DNA binding activity in these complexes. Subunits p65 and p50 were also detected in the high mobility complex observed 4 days after weaning (Fig. 1B). We suggest that this may contain proteolytically cleaved forms of NF-κB.

To confirm the activation of NF-κB during involution, we performed immunohistochemistry on paraffin-embedded sections of mammary glands using an antibody to NF-κB p65. Weakly diffuse staining of epithelium and stromal tissue was observed in sections from day 10 lactating mice (Fig. 2A). At 24 h of involution, strong nuclear staining was evident in approximately half of the luminal epithelial cells (Fig. 2, B and E). These positively stained cells exhibited a normal nuclear intensity throughout the time course. The more slowly migrating species consisted almost exclusively of p65 and p50 subunits, whereas the faster migrating species consisted of p50 but little or no p65 (Fig. 1B). Additional isoforms other than p52 (Fig. 1B) may be responsible for residual DNA binding activity in these complexes. Subunits p65 and p50 were also detected in the high mobility complex observed 4 days after weaning (Fig. 1B). We suggest that this may contain proteolytically cleaved forms of NF-κB.

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morphology and were distributed evenly along the intact epithelial lining of the lobulo-alveolar compartment (Fig. 2, B and E). Similar staining was observed in sections of mammary gland harvested at 72 h of involution. In addition to the reorganization of the lobulo-alveolar structure of the gland, there was an apparent increase in the proportion of positively stained nuclei in the surviving epithelial population (Fig. 2, C and F). These data demonstrate that activation of NF-κB occurs in nonapoptotic epithelial cells of the mammary gland during involution.

**NF-κB Activity in KIM-2 Cells**—DNA binding activity of NF-κB in KIM-2 mammary epithelial cells cultured in vitro was concordant with the data observed during gestation, lactation, and involution of mammary glands in vivo (Fig. 3A). Proliferating, undifferentiated cells exhibited elevated levels of NF-κB activity, which reduced upon growth arrest and became undetectable following incubation for 6 days in DM, which contained the lactogenic hormones prolactin and dexamethasone. At this time, expression of whey acid protein, a late marker of differentiation, was induced (32). We have previously shown that differentiated KIM-2 cells rapidly undergo apoptosis following removal of the lactogenic hormones from the culture medium (32). Under these conditions (with AM), NF-κB activity became markedly elevated. Detailed inspection of the kinetics of this response revealed that DNA binding activity was highest after a 4-h incubation in AM and remained above unstimulated levels for up to 96 h (Fig. 3B). Maximal NF-κB activity preceded detectable annexin V staining in situ (data not shown). Two complexes were observed (Fig. 3B, i and ii) and, in accordance with mammary gland extracts, these complexes were shown to consist predominantly of p65/p50 (more slowly migrating species) and p50 (faster migrating species).
versus B). All three ligands also suppressed the levels of GFP (Fig. 5).

To determine whether NF-κB suppressed apoptosis in KIM-2 cells, Annexin V and GFP did not co-localize to the same cells (Fig. 6). Cells were stained with annexin V and counted by flow cytometry (data not shown). To ascertain whether this increased DNA degradation, whereas cytosolic p65 levels were comparable to the NF-κB responsive promoters, we treated NGR-KIM-2 clones, or maintained in DM (panels i and ii) for 17 h, stained with annexin V (red), and visualized by fluorescence microscopy at ×20 magnification. B, KIM-2 cells were treated with medium (AM or DM) for 17 h in the presence of PBS, IL-1α (n = 6), TNF-α (n = 7), or LPS (n = 7). Cells were stained with annexin V and counted by flow cytometry. The graph represents the percent of cell population staining for annexin V only. t tests: *, AM versus AM + LPS, p < 0.001; **, AM versus AM + TNF-α, p < 0.001; ***, AM versus AM + IL-1α, p < 0.001.

isoforms (Fig. 1B). Both isoforms exhibited similar changes in DNA binding activity. This activity also coincided with a decrease in the cytoplasmic levels of IκBα protein indicative of IκBα degradation, whereas cytosolic p65 levels were comparably stable (Fig. 4).

**Activation of NF-κB Suppressed Apoptosis in KIM-2 Cells**—To determine whether NF-κB influenced the apoptotic response induced by hormonal withdrawal, we stimulated NF-κB in KIM-2 cells via three distinct pathways with the soluble ligands LPS, IL-1α, and TNF-α. All three ligands stimulated a rapid and prolonged activation of NF-κB DNA binding (data not shown). To ascertain whether this increased DNA binding activity resulted in increased trans-activation of NF-κB responsive promoters, we treated NGR-KIM-2 clones, stably transfected with an NF-κB-responsive GFP reporter construct, with either TNF-α or AM. Both treatments resulted in an increase in the proportion of cells expressing detectable levels of GFP (Fig. 5A). All three ligands also suppressed the extent of apoptosis normally observed in KIM-2 cells following hormone withdrawal (Fig. 5B). Thus, all three ligands that induced NF-κB in KIM-2 cells partially compensated for the removal of survival factors from the medium.

In KIM-2 cells induced to die by removal of lactogenic hormones, annexin V and GFP did not co-localize to the same cells (Fig. 5A). In an analysis of over 1000 annexin V-positive cells *in situ*, we did not detect a single example of co-localized GFP and annexin V, suggesting that NF-κB was activated selectively in surviving cells.

To circumvent the possibility of secondary effects of these ligands on the cell death pathways, we introduced an antisense adenosinoviral expression construct for IκBα, the endogenous repressor of NF-κB, into KIM-2 cells. Infection of confluent monolayers of differentiated KIM-2 cells by this method resulted in approximately 30% transduction efficiency as measured by β-galactosidase assay (data not shown) using an adenosinoviral LacZ expression construct (adV-LacZ), which was also used as a control vector for the adenosinoviral antisense IκB construct (Fig. 6, A and B). Expression of the antisense construct (adV-asIκB) potentiated both NF-κB DNA binding in KIM-2 cells as shown by EMSA (Fig. 6A) and transcriptional activation of the NF-κB-responsive promoter in NGH-KIM-2 cells (Fig. 6B). Indeed, induction of NF-κB by the antisense IκB construct exceeded the previously observed NF-κB response to the apoptotic stimulus (AM) alone (Fig. 6A).

Differentiated KIM-2 cells, transduced with adV-asIκB or adV-LacZ constructs, were tested for their response to the removal of lactogenic hormones (Fig. 6C). Cells were subjected to AM for 17 h and then assessed for apoptosis by annexin V staining and flow cytometry. The enhanced activation of NF-κB with as IκB inhibited the induction of apoptosis following removal of lactogenic hormones (Fig. 6B), confirming the results obtained with cytokine stimulation. The inhibitory effect was greater than that achieved by co-incubation with the ligands (Fig. 5A).
NF-κB Inhibits Death in Mammary Epithelia

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Modulation of apoptosis is now a well recognized function of NF-κB. In addition to many reports of its activation by cytotoxic and pro-apoptotic insults including DNA damage, cytokines, and hypoxia (4, 5), there is some evidence to suggest that this transcription factor also regulates apoptosis during fetal development (8, 37). However, our understanding of its precise role in developing tissues is rudimentary. In this study we have demonstrated a novel, stage-specific pattern of NF-κB activity during adult mammary gland development and have also shown a direct effect of this transcription factor on epithelial cell death.

NF-κB was activated during involution and was restricted to mammary epithelia, suggesting that it may directly regulate luminal epithelial cell death as part of the normal developmental cycle. Activated NF-κB consisted predominantly of p65 and p50 subunits, which are the most commonly activated forms of NF-κB in other cell types (4, 5). This is one of a growing number of transcriptional regulators known to be activated within the first 24 h of involution (15, 17, 20–24), signifying a substantial reorganization of gene expression in the gland during the transition from lactation to involution. Activation of NF-κB is one of the most rapid transcription factor responses, with detectable DNA binding within 2 h following cessation of suckling and significantly greater activity observed after 24 h. Two events are known to occur sufficiently early during involution to potentially cause this activation. Milk stasis occurs in the gland as a result of the cessation of milk removal; this is thought to induce apoptosis, possibly through mechanical stretching of the secretory epithelium lining the alveoli of the gland (35). A similar mechanical stimulus activates NF-κB in vascular endothelial cells (38). In addition to milk stasis, a decline in the levels of lactogenic hormones promotes the onset of involution (15, 20, 35). Whether the removal of a hormonal stimulus could directly activate NF-κB activity remains speculative. However, this possibility is supported by our in vitro model of hormone-dependent survival of KIM-2 cells in which apoptosis and NF-κB are induced following removal of lactogenic hormones (Fig. 3 and Ref. 32).

In this in vitro model, approximately one-third of the cells become apoptotic after 17 h of hormone depletion. In contrast, just 3% of the epithelial population of the mammary gland appeared apoptotic after 24 h involution (23). A number of factors could explain this apparent disparity, such as a deficiency of stromal and extracellular matrix interactions in cell culture, the effect of sudden and complete removal of hormones from the medium in vitro, and the fact that apoptotic cells are rapidly cleared in vivo by phagocytosis.

We have shown that IκBα levels decline as NF-κB activity increases in KIM-2 cells, suggesting that NF-κB is activated in mammary epithelium by targeted degradation of this repressor. The source of the stimulus remains elusive, but several signaling pathways that result in phosphorylation and degradation of IκBα have been described in mammary epithelial cells (25). In addition to the mechanical stretch stimulus and peptide hormone signals described above, the ability of TNF-α and IL-1α to induce NF-κB in KIM-2 cells suggests that cytokines are able to activate NF-κB in epithelium in vivo as described for other cell types (9–12). Cytokines are present in milk and are proposed to act in a paracrine/autocrine fashion to influence mammary function (39). Potential sources of these cytokines include milk leukocytes (40), mammary epithelium (41), and adipocytes of the stroma (42).

Our data unequivocally show that NF-κB suppresses apoptosis in mammary epithelial cells and give the first example of a transcription factor mediating survival in mammary involution. Thus, activation of NF-κB with extracellular ligands (Fig. 5) or by specific inhibition of the endogenous repressor IκBα (Fig. 6) led to suppression of apoptosis in KIM-2 cells. In the latter case, almost all apoptosis was blocked, indicating that NF-κB can compensate for the loss of all three survival factors: insulin, prolactin, and dexamethasone. The absence of active NF-κB in apoptotic cells both in vivo (Fig. 2) and in vitro (Fig. 6B) further supports our conclusion that NF-κB promotes survival during the involution process. Paradoxically, our results using two separate assays indicated that NF-κB activity was negligible during lactation when the frequency of apoptosis was low (Figs. 1 and 2). Similarly, NF-κB activity in differentiated KIM-2 cells was barely detectable (Fig. 3). We conclude that signaling molecules other than NF-κB mediate survival of differentiated epithelium during lactation and that NF-κB is induced, either directly or indirectly, by an apoptotic stimulus that is initiated immediately following pup withdrawal. This unexpected observation suggests that internal homeostatic signals exist within epithelium to modulate involution by selectively conserving certain cells in the population.

This hypothesis is consistent with the paradigm of NF-κB-mediated suppression of TNF-α cytotoxicity in TNF-α-responsive cells (9–12). TNF-α stimulates NF-κB through targeted degradation of IκB. NF-κB then inhibits the death signal by trans-activating genes that promote resistance to apoptosis (9). The effect of this negative feedback mediated by NF-κB is the modulation of apoptosis in response to the TNF-α death signal.

A modulatory function of NF-κB is also consistent with the current two-stage model of mammary gland involution (16, 20). The first stage of involution is initiated by unknown, locally derived stimuli and is accompanied by activation of Stat3 (11, 43), Bcl-XL, and Bax (44), down-regulation of the milk protein genes and Stat5 (11, 43), and an increase in the number of epithelial cells undergoing apoptosis (15, 16, 20). After approximately 48 h, the gland begins the second stage of involution, characterized by an increase in metalloproteinase gene expression, degradation of the supporting basement membrane, and subsequent remodeling of the gland (15, 16). Initial activation of NF-κB during first stage involution could be a direct result of perturbation of cell contacts between apoptotic cells and their neighbors. Our cell model allows us to look at apoptotic responses in the context of cell adhesions and extracellular matrix interactions (32). Preliminary data suggest that KIM-2 cells exhibit morphological and cell adhesion changes in response to apoptosis in adjacent cells. A similar process has been demonstrated in kidney cortex epithelium, where cells rearrange and alter the distribution of cell adhesion molecules as a consequence of their contact with apoptotic neighbors (45). NF-κB activity is maximally induced at the onset of second-phase involution, at a time when proteases disrupt cell-extracellular matrix interactions (16). Similarly, the loss of extracellular matrix contacts in cultured pancreatic acinar cells leads to NF-κB activation and apoptosis (46). These findings raise the speculative but intriguing possibility that NF-κB is induced as a direct consequence of a reduction in adhesion-mediated cell survival and is consistent with our proposal that NF-κB performs a homeostatic function during involution. This hypothesis is supported by the fact that NF-κB regulates a
number of integrins and cell adhesion molecules that are known to promote mammary epithelial cell survival (18, 25).

Given its apparent role in mammary gland, inappropriate NF-κB signaling may lead to the progressive accumulation of cells that would otherwise be destined to undergo apoptosis. Therefore, constitutive NF-κB activity could exacerbate mammary breast cancer. Indeed, previous studies have described elevated levels of NF-κB in mammary tumors (26–28). However, the conclusions reached by these studies relating to the identity of the subunits activated and, consequently, the precise function of NF-κB, were inconsistent. In contrast, we show here an increase of NF-κB in normal tissue during adult mammary development, and we show that this correlates with trans-activation of an NF-κB responsive promoter and suppression of death. Our results, therefore, provide a mechanism by which high levels of NF-κB may contribute to tumor progression, pointing to NF-κB as a future target of therapeutics for breast cancer.

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