Caspases: Decoders of Apoptotic Signals During Mammary Involution

Caspase activation during involution

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Abstract: At weaning most of the alveolar epithelial cells in the mammary gland die by apoptosis and are removed by phagocytosis. Caspases are a family of aspartate specific cysteine proteases. Activation of caspases is generally thought to represent a major and irreversible event in the apoptotic process. We analyzed caspase expression and activation during mammary gland involution. A quantitative RT-PCR based approach revealed that levels of mRNA expression of several caspases are induced during involution. Using an antibody that specifically recognizes activated caspases we measured a transient induction of caspase activity *in situ* and found a maximal activation at days two and three of involution. These data were corroborated by monitoring caspase-3 like activity in mammary extracts with a synthetic DEVD-afc peptide as caspase-3 substrate. Using Fas-deficient mice we present evidence that the Fas signaling pathway is not essential for caspase activation and apoptosis during mammary gland involution. In summary, signaling pathways during involution seem to involve activation of caspases as intraepithelial triggers of mammary epithelial cell apoptosis.

1. INTRODUCTION

Proliferation and programmed cell death (PCD) are two major principles contributing to many different biological processes during development and in adults. Apoptosis is a form of PCD which is morphologically characterized by membrane blebbing, condensation of chromatin and generation of apoptotic bodies consisting of membrane surrounded cellular fragments[1]. In many cases it is an energy dependent process requiring gene expression and a non-random fragmentation of genomic DNA is often observed during apoptosis[1, 2]. Induction of apoptosis involves the activation of specific death signaling pathways including the activation of proteases termed caspases. Caspases are a family of aspartate specific cysteine-proteases that are believed to be major effectors of apoptosis [3, 4]. They share typical structural and functional features such as an N-terminal pro-domain that is variable in length and sequence and two catalytic subunits. The prodomain mediates subcellular localization and protein/protein interaction; the catalytic subunits include a large ~17-20 kDa and a small ~10 kDa form. During apoptosis caspases are activated by a proteolytical separation of the large and small subunits that assemble to form the active enzyme complex. Many caspase substrates are cleaved by limited proteolysis, among them are poly(ADP-ribose) polymerase (PARP)[5], α-fodrin[6], MEKK-1[7], U1 associated 70 kDa protein[8], DNA fragmentation factor (DFF)[9, 10] and Bcl-2[11].

In the mammary gland apoptosis occurs in secretory epithelial cells during post-lactational involution[12,13]. Upon removing the pups from their mother, milk transiently accumulates in the glands resulting in a strong engorgement. At about three days of involution, milk is resorbed, alveolar structures collapse and epithelial cells undergo apoptosis. The lack of the suckling stimulus also leads to a drop of lactogenic hormone levels (e.g. prolactin and glucocorticoids) which is believed to contribute to the involution process in all glands[14,15]. Accumulation of milk and changes of hormone levels are possibly major triggers of apoptosis[15, 16, 17].

Several signal transduction pathways resulting in caspase activation have been described that involve death receptors such as Fas/APO-1[18]. Indeed, for induction of apoptosis in the prostate and the ovary it was proposed that activation of Fas may play an important role[19, 20]. These studies mainly relied on animals with defined genetic mutations in the Fas pathway[21]. Here we studied the involvement of the Fas pathway and caspases during involution of the mouse mammary gland. After weaning, elevated caspase-3 and caspase-9 mRNA expression and an induction of caspase activity were observed. In Fas-deficient mice (lpr mice) caspase activation and apoptosis as analyzed by terminal transferase assays (TUNEL) was indistinguishable

from wildtype animals implicating that the Fas signaling pathway is not essential for mammary gland involution.

2. RESULTS AND DISCUSSION

For the mammary gland, little information is available about expression and activity of caspases during phases of epithelial cell apoptosis. For caspase-1 an induction at the mRNA level was shown during involution [22,23]; in addition, Krajewska and co-workers[24] demonstrated the presence of caspase-3 protein in ductal epithelial cells of human mammary biopsies. We investigated the expression of caspase-3 and caspase-9 in the mammary gland by quantitative RT-PCR (using Taqman technology). Total RNA of mammary tissue at lactation and 2, 3, 6, and 10 days of involution was prepared and Taqman-PCR was performed. As an internal reference 7S ribosomal RNA was measured. In addition, c-*fos*, a gene that was previously shown to be induced during involution[25], was also analyzed. After weaning, an induction of caspase-3 and caspase-9 mRNA expression was observed.

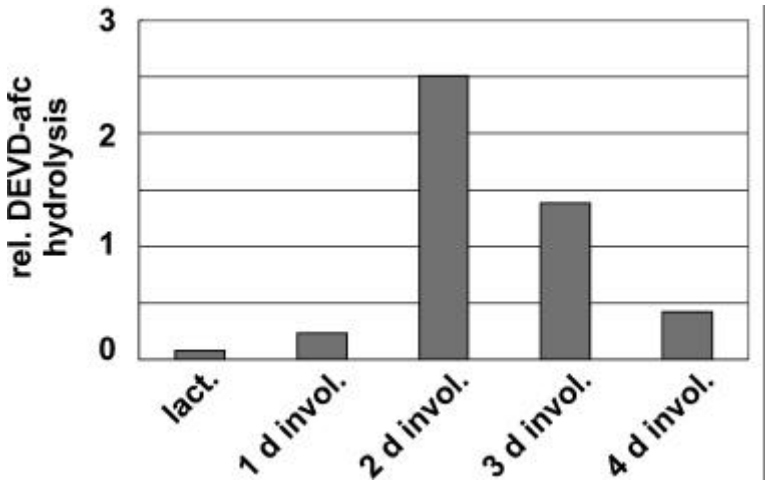

Figure 1. Caspase cleavage activity during lactation and involution. Cytoplasmic extracts were prepared from mammary glands at lactation and 1, 2, 3 and 4 days of involution as described[25]. 25 µg protein were incubated in the presence of DEVD-afc in caspase buffer (Promega) and fluorescence was measured in a Fluorometer over a period of 1 hour (200 cycles of measurement). Shown are the relative DEVD cleavage activities as arbitrary units of fluorescence.

Induction of c-*fos* and caspase-3 mRNA expression was maximal at day 2 (35 fold induction for c-*fos*, 3.3 fold induction for caspase-3) whereas

caspase-9 mRNA levels were maximal at day 6 of involution (10.5 fold induction). These results indicate that, like caspase-1, caspase-3 and caspase-9 mRNA levels are induced during mammary gland involution.

To further investigate the involvement of caspases during involution, caspase activity was analyzed in mammary extracts derived of glands at lactation and 1, 2, 3, and 4 days of involution. Extracts were incubated with a synthetic caspase substrate (DEVD-afc) and cleavage of the substrate was analyzed in a fluorometer.

Figure 1 shows that caspase activity was strongly and transiently induced, peaking at day 2 of involution. Caspase activation was further studied on mammary tissue sections using an antibody that specifically recognizes active caspase-3 but not the inactive zymogen (CM1 antibody)[26]. As shown in Figure 2, a strong induction of active caspase-3 was observed in epithelial cells at 3 days of involution (panel B) whereas almost no active caspase was found in epithelial cells at lactation (panel A).

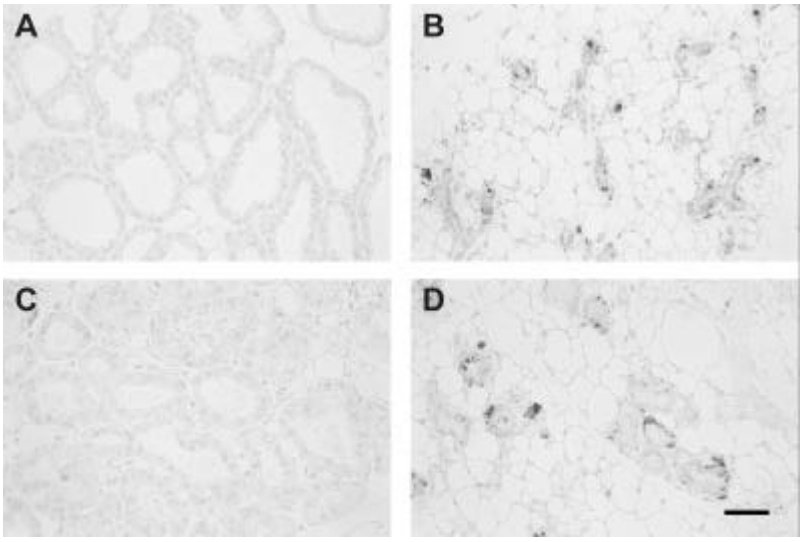

Figure 2. Caspase activation during involution. Shown are mammary glands of wildtype (panels A, B) and Fas-deficient mice (panels C, D) at lactation (panel A, C) and at 3 days of involution (panel B, D). Paraffin embedded sections from paraformaldehyde fixed samples were incubated with an antibody (CM1) that recognizes active caspase-3[26]. Antigen-antibody complexes were detected with the EnVision System (DAKO, Glostrup, Denmark) using AEC as substrate. The bar represents 25 μm.

In order to investigate a potential involvement of Fas during mammary gland involution, Fas-deficient mice were analyzed immunohistochemically for caspase-3 like activity at lactation (Figure 2, panel C) and at 3 days of involution (panel D). A similar caspase activation was observed in Fas-deficient animals as compared to wildtype animals.

These results indicate that caspases are activated during involution in the absence of functional Fas. To confirm these data, mammary sections derived of wildtype and Fas-deficient animals were analyzed for DNA fragmentation using a terminal transferase (TUNEL) assay (Figure 3). Both, in wildtype and in Fas-deficient animals similar levels of DNA fragmentation were found at 2 days of involution (panels B, D) whereas almost no cells stained positive for fragmented DNA at lactation (panels A, C). In addition, total DNA was isolated from wildtype and Fas-deficient animals at lactation and 3 days of involution and analyzed by gel electrophoresis (data not shown). Again, the typical DNA ladder was observed in wildtype and in Fas-deficient animals at 2 and 3 days of involution but not at lactation. Taken together, these results indicate that mammary epithelial cell apoptosis occurs to a similar extent in Fas-deficient mice and in wildtype animals.

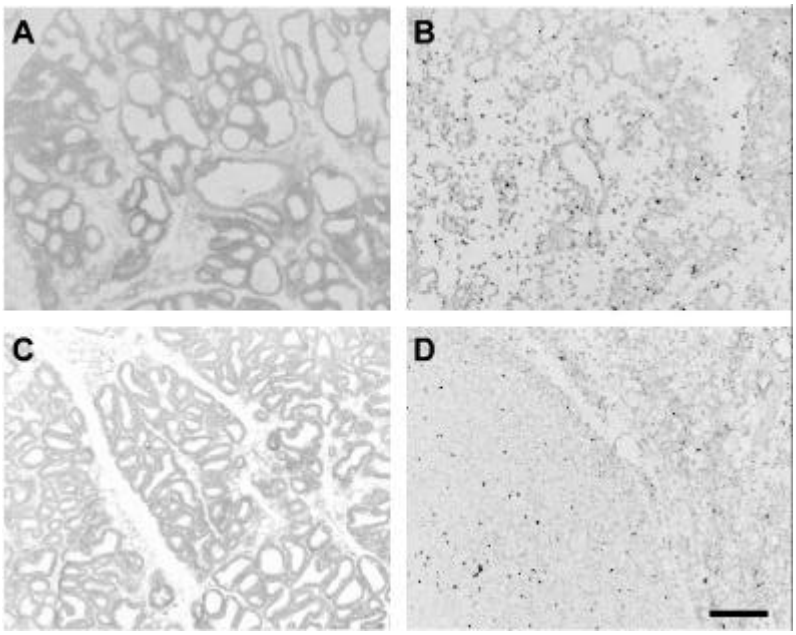

Figure 3. DNA fragmentation during involution. Terminal transferase assays (TUNEL) were performed on mammary gland sections of wildtype (panels A and B) and Fas-deficient animals (panels C and D) at lactation (A and C) and at 2 days of involution (B and D). The bar represents 50 μm.

In summary, our data suggest that epithelial cell death that occurs during mammary gland involution involves an activation of caspases, both, at the mRNA and protein level. Cell death in the mammary gland seems to occur in a Fas independent manner.

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