MOD 1810

ELSEVIER

Mechanisms of Development xx (2001) xxx–xxx

www.elsevier.com/locate/modo

Mouse mammary gland involution is associated with cytochrome *c* release and caspase activation

Andreas Marti[a,*], Philipp M. Ritter[b], Richard Jäger[c], Hedvika Lazar[a], Anna Baltzer[a], Johannes Schenkel[d], Wim Declercq[e], Peter Vandenabeele[e], Rolf Jaggi[a]

[a]*Department of Clinical Research, University of Bern, Murtenstrasse 35, 3010 Bern, Switzerland*
[b]*Swiss Federal Dairy Research Station, Schwarzenburgstrasse 157, 3003 Bern-Liebefeld, Switzerland*
[c]*Research Center Karlsruhe, Institute for Toxicology and Genetics, Hermann-von-Helmholtz-Platz 1, D-76344 Eggenstein-Leopoldshafen, Germany*
[d]*Institute of Physiology and Pathophysiology, University of Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany*
[e]*Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology (VIB) and University of Ghent., K.L. Ledeganckstraat 35, 9000 Ghent, Belgium*

Received 9 February 2001; received in revised form 11 April 2001; accepted 11 April 2001

Abstract

At weaning, milk producing mammary epithelial cells undergo apoptosis and are removed by phagocytosis. Here, we show that mouse mammary gland involution is associated with mitochondrial cytochrome *c* release and processing of numerous caspases, including caspase-1, -3, -7, -8 and -9. Induction of caspase-3-like activity paralleled cleavage of poly-(ADP–ribose) polymerase. Dexamethasone inhibited processing of caspase-3, -7 and -8 and apoptosis, but had no effect on caspase-1 accumulation and cytochrome *c* release. In Bcl-2 transgenic animals, cytochrome *c* release, caspase activation and apoptosis were impaired. Thus, the pro-apoptotic signaling pathway in mammary epithelial cells during involution involves the release of cytochrome *c* and activation of caspases. It is inhibited by Bcl-2 at the mitochondrial level and by dexamethasone at a post-mitochondrial level.

Keywords: Apoptosis; Caspase; Cytochrome *c*; Involution; Programmed cell death

1. Introduction

Programmed cell death or apoptosis is an essential process for organogenesis, tissue homeostasis, development and function of the immune system and the nervous system. Deregulated apoptosis contributes to many severe diseases, like AIDS, cancer and many forms of degeneration. Originally described by pathologists on a morphological basis, apoptosis is characterized by cell shrinkage and chromatin condensation, followed by the formation of membrane-bound nuclear and cellular fragments (Kerr et al., 1972). Most significantly, since cellular membranes remain intact, an inflammatory response is avoided during apoptosis (Wyllie et al., 1980; Vaux, 1993; Wyllie, 1995). As a process, apoptosis can be structured into the reversible induction phase, the irreversible execution phase and a clean-up phase in which the cellular fragments are disposed (Vaux and Strasser, 1996).

A family of intracellular proteases, termed caspases, has been recognized as being essential for the rapid progression through apoptosis and for the exhibition of the typical apoptotic morphology. Caspases are aspartate-specific proteases with a catalytic cysteine in the active site (Alnemri et al., 1996; Salvesen and Dixit, 1997; Kidd, 1998; Thornberry and Lazebnik, 1998). Activation of caspases involves hetero- or auto-proteolytic processing of pro-caspases, usually at aspartate residues, to give rise to active caspase fragments that join to form an active caspase. To date, over 50 caspase substrates beside the caspases themselves have been implicated in various cell systems. They include such diverse proteins as DNA repair enzymes, structural proteins and protein kinases (Kidd, 1998; Porter and Jänicke, 1999).

A major breakthrough in the understanding of how caspases are activated was the discovery that during apoptosis, cytochrome *c* was released from mitochondria into the cytosol, where it acts as a potent activator of caspases (Liu et al., 1996; Li et al., 1997b; Zou et al., 1997). Cytochrome *c* release can be induced by a variety of pro-apoptotic signals, such as death receptor-mediated signaling, ionizing radiation, chemotherapeutic agents and kinase inhibitors such as

* Corresponding author. Tel.: +41-31-632-40-37; fax: +41-31-632-32-97.
E-mail address: andreas.marti@dkf4.unibe.ch (A. Marti).

0925-4773/01/$ - see front matter
PII: S0925-4773(01)00381-1

staurosporine (Green and Reed, 1998). Once released from mitochondria, cytochrome *c* forms a multimeric complex with the apoptosis-activating factor, Apaf-1, that recruits and activates pro-caspases (e.g. pro-caspase-9) to generate a functional apoptosome (Li et al., 1997b; Zou et al., 1997; Cecconi, 1999). The role of the apoptosome during apoptosis has been well-described in cells in culture. Studies in vivo have been hampered by the fact that animals deficient in cytochrome *c*, Apaf-1 or caspase-9 die very early during development or after birth (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998; Li et al., 2000). However, the Apaf-1 and the caspase-9 knockout studies both indicate an important role for the apoptosome during nerve cell development and, in addition, Apaf-1 knockout animals display a delayed development of digit formation. To our knowledge, there is presently no study that has directly addressed the role of cytochrome *c* during developmentally controlled cell death, such as during digit formation, nerve cell death in newborns, apoptosis in the ovary, or involution of the mammary gland after lactation.

Different protein families have been implicated directly or indirectly in regulating cytochrome *c* release or caspase activity, such as members of the Bcl-2/Bax family of proteins (Green and Reed, 1998), competitive FLICE-like inhibitory proteins (FLIPs; Tschopp et al., 1998) and inhibitor of apoptosis proteins (IAPs; Deveraux and Reed, 1999). Members of the Bcl-2/Bax family are membrane-localized and may function as membrane pores (Reed, 1997, 1998). A major site of action of Bcl-2 members seems to be the mitochondrial membrane (Green and Reed, 1998). Anti-apoptotic members of the Bcl-2 family, like Bcl-2 itself, have the ability to inhibit, whereas pro-apoptotic members, i.e. Bax, promote cytochrome *c* release from mitochondria (Reed, 1998).

Mammary gland development is characterized by proliferation of the epithelium during puberty, pregnancy and during each estrus cycle, and by terminal differentiation of secretory epithelial cells and milk production during late pregnancy and lactation. After the lactation period, the gland undergoes an extensive remodeling process that leads to the involution of epithelial structures until a state is reached resembling that of a virgin gland (Walker et al., 1989; Strange et al., 1992). 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., 1994, 1999b). 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 (Ossowski et al., 1979; Topper and Freeman, 1980; Feng et al., 1995; Li et al., 1997a; Marti et al., 1997; Chapman et al., 1999; Marti et al., 1999b). 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 (ECM) 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., 1997a).

We previously showed that mammary epithelial cell apoptosis at weaning is associated with caspase activity (Marti et al., 1999a). Here, we studied the release of cytochrome *c* as a possible trigger of caspase activation in vivo in the involuting mouse mammary gland. We present evidence that processing and possible activation of numerous caspases, including caspase-1, -3, -7, -8 and -9, and cleavage of caspase substrates, such as poly-(ADP–ribose) polymerase (PARP), are paralleled or preceded by a release of mitochondrial cytochrome *c* in the involuting gland. Overexpression of Bcl-2 in the mammary gland results in an inhibition of cytochrome *c* release, caspase processing and PARP cleavage. Inhibition of involution by implantation of dexamethasone-release pellets into glands similarly impairs caspase processing and apoptosis, but not the release of cytochrome *c* from mitochondria, suggesting that dexamethasone inhibits involution by blocking caspase activation at a step downstream or independent of mitochondrial cytochrome *c* release.

2. Results

2.1. Involvement of cytochrome c and caspase-9 during mammary gland involution

We investigated the potential role of cytochrome *c* and caspases for epithelial cell apoptosis during mouse mammary gland involution. Mitochondria-enriched samples were prepared from glands of MORO mice at lactation or at 1, 2 and 3 days after removing the pups, and cytochrome *c* was analyzed by Western blot analysis (Fig. 1, panel A). The levels of cytochrome *c* strongly decreased during involution (lanes 2–4) as compared with lactation (lane 1). In contrast, the levels of mitochondrial cytochrome oxidase IV (COX IV) decreased only moderately from lactation to day 3 of involution (panel B, lanes 1–4) in these mitochondria-enriched fractions.

Pro-caspase-9 is activated by binding to the cytochrome *c*/Apaf-1 complex (Li et al., 1997b) that forms after cytochrome *c* is released by mitochondria. Activation of pro-caspase-9 is paralleled by a processing of the 49 kDa zymogen to a large and a small subunit of about 20 and 10 kDa, respectively. We measured caspase-9 in cytoplasmic mammary extracts with two different antibodies, one reacting preferentially with pro-caspase-9 zymogen and one reacting with the large subunit of about 20 kDa. Panel C shows that the levels of pro-caspase-9 protein decreased during involution (lanes 2–4) as compared with the levels at lactation (lane 1). The loss of caspase-9 zymogen during involution was paralleled by an accumulation of the

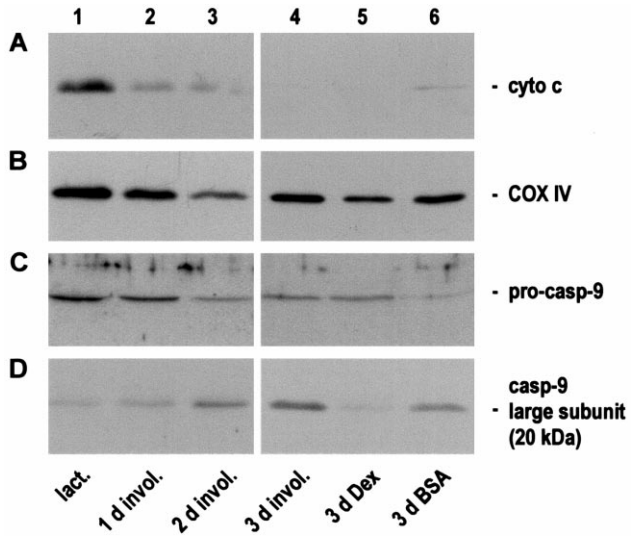

Fig. 1. Cytochrome *c* release and caspase-9 processing during normal mouse mammary gland involution and in dexamethasone-exposed glands. Extracts were prepared from mammary glands at lactation (lane 1), and at 1 (lane 2), 2 (lane 3) and 3 days of normal involution (lane 4), and 3 days after dexamethasone (lane 5) or control BSA-pellet implantation (lane 6). Western blot analyses were performed with mitochondria-enriched fractions (panels A and B) and cytoplasmic extracts (panels C and D) using antibodies specific for cytochrome *c* (cyto c; panel A), COX IV (panel B), and caspase-9 (panels C and D).

processed 20 kDa subunit (panel D). These results indicate that mammary epithelial cell death during involution is associated with mitochondrial loss of cytochrome *c* and activation of pro-caspase-9.

2.2. Effects of dexamethasone on cytochrome c release and caspase-9 processing during mammary gland involution

We previously described that dexamethasone-releasing plastic pellets inhibit involution when implanted into the lactating mammary gland (Feng et al., 1995). To test whether dexamethasone inhibits cytochrome *c* release, we implanted dexamethasone-release plastic pellets into the fourth inguinal gland of lactating mice. Fig. 1 documents that the levels of cytochrome *c* were decreased in mitochondria-enriched fractions of dexamethasone-treated glands (panel A, lane 5) similar to 3 days of involution in untreated glands (lane 4). Likewise, implantation of control pellets containing only the carrier protein bovine serum albumin (BSA) had no effect on the cytochrome *c* release 3 days after pellet implantation and removal of the pups (lane 6). As a control for the presence of mitochondria, the level of COX IV remained comparable in mitochondria-enriched fractions at all conditions (panel B, lanes 4–6). In the presence of dexamethasone, pro-caspase-9 expression levels were reduced to the levels of untreated glands at 3 days of involution (panel C, compare lane 4 with lane 5). However, the appearance of the small 20 kDa caspase-9 subunit was significantly impaired by dexamethasone (panel D, lane 5). Implantation of BSA control pellets had no effects on the accumulation of processed caspase-9 subunits during involution (compare lane 4 with lane 6). These results indicate that dexamethasone may affect directly or indirectly the processing of pro-caspase-9.

2.3. Effects of dexamethasone on caspase processing and activity during involution

To further study the effect of dexamethasone on caspases, additional members of the caspase family of proteins were analyzed. Caspase analyses were performed in cytoplasmic and nuclear extracts prepared from mammary glands. A direct substrate of caspase-9 is pro-caspase-3, which represents a classical executioner caspase, similar to caspase-7 (Kidd, 1998). Fig. 2 shows the protein levels of caspase-3 (panel A) and caspase-7 (panel B) in cytoplasmic extracts prepared from glands at lactation (lane 1), normal involution days 1–3 (lanes 2–4) and at 3 days of involution after implantation of dexamethasone-release (lane 5) or BSA control pellets (lane 6). Pro-caspase-3 and pro-caspase-7 were expressed during lactation (lane 1) and protein levels were reduced during involution (lanes 2–4). No processed fragments of caspase-3 could be detected in mammary extracts derived from involuting glands with the available antibody. Processed fragments of caspase-7 were detected during involution in both cytoplasmic and nuclear fractions, however, the appearance of mature processed forms and intermediate forms varied from animal to animal (data not shown). Implantation of dexamethasone-release pellets prevented processing of pro-caspase-3 and pro-caspase-7 (lane 5). In contrast, BSA control pellets did not prevent caspase processing (lane 6).

Caspase-8 acts as an initiator caspase, e.g. in the Fas and TNF receptor pathways (Boldin et al., 1996; Muzio et al., 1996). However, caspase-8 can also be activated as a late event in the cytochrome *c* pathway (Slee et al., 1999). We found a partial processing of pro-caspase-8 at day 3 of involution (panel C, lane 4) which was completely prevented in dexamethasone-exposed glands (lane 5), but not in BSA control pellet-implanted glands (lane 6). Cleavage of caspase-8 seems to be a relatively late event since no processed caspase-8 fragments were detectable in mammary glands at day 1 (lane 2) or day 2 (lane 3) of involution.

The level of caspase-1 proform increased during involution (panel D, short exposure) and partially processed caspase-1 fragments were detected from day 1 of involution (panel E, lane 2, long exposure). At day 3 of involution, caspase-1 processing was even more pronounced with the appearance of a 26 kDa subunit (panel E, lane 4). Although dexamethasone could not prevent the accumulation of pro-caspase-1 protein (panel D, lane 5), it inhibited the accumulation of the 26 kDa form (panel E, lane 5). Caspase-11 has been reported to be responsible for activation of pro-caspase-1 (Wang et al., 1998). No signal was detected in mammary gland extracts using a caspase-11-specific antibody (data not shown).

The results described above indicate that dexamethasone-exposure of mammary epithelial cells during involution impairs apoptosis by inhibiting caspase processing at a level downstream or independent of the mitochondrial efflux of cytochrome *c*. In order to monitor caspase activity, we analyzed nuclear PARP (panel F), a well-characterized caspase substrate that was reported to be processed during apoptosis by caspase-mediated cleavage (Rosen and Casciola-Rosen, 1997). PARP was clearly processed at days 2 and 3 of involution (lanes 3 and 4), but not at lactation (lane 1). PARP processing was inhibited by dexamethasone (lane 5), whereas BSA control pellets were without effect (lane 6). Although several different PARP-specific antibodies detected a p85 PARP cleavage product in apoptotic human and mouse cells in culture (data not shown), we never detected the expected p85 product in vivo in the involuting mammary gland.

We monitored caspase activity in mammary extracts by measuring the cleavage of a synthetic Asp-Glu-Val-Asp–amino-methyl-coumarin (DEVD–amc) peptide predominantly recognized by caspase-3. Essentially, the same results were obtained with cytoplasmic and nuclear extracts of lactating and involuting glands. However, in nuclear extracts, the caspase activity was consistently stronger. Caspase-mediated DEVD–amc cleavage results in the release of fluorescent amc that can be measured in a fluorometer. As shown in panel G, DEVD–amc cleavage activity (depicted as relative activity during 50 min of incubation) was low in nuclear extracts prepared from glands during lactation (lane 1) and was transiently increased during involution, reaching maximal activity at day 2 (lanes 2–4). Implantation of dexamethasone-release pellets resulted in a complete inhibition of caspase activation (lane 5), whereas the induction of caspase cleavage activity in BSA-pellet-implanted glands (lane 6) was comparable with untreated glands at 3 days of involution (lane 4). A peptide that is cleaved by caspase-9 (LEHD–afc) was also tested in cytoplasmic and nuclear extracts. Similar results were obtained as with the DEVD–amc peptide (data not shown).

The CM-1 antibody was previously shown to selectively detect active caspase-3 on paraffin sections (Srinivasan et al., 1998). We used the CM-1 antibody on sections of mammary glands at lactation (Fig. 3, panel A) and 3 days after weaning (panel B); or of mammary glands 3 days after

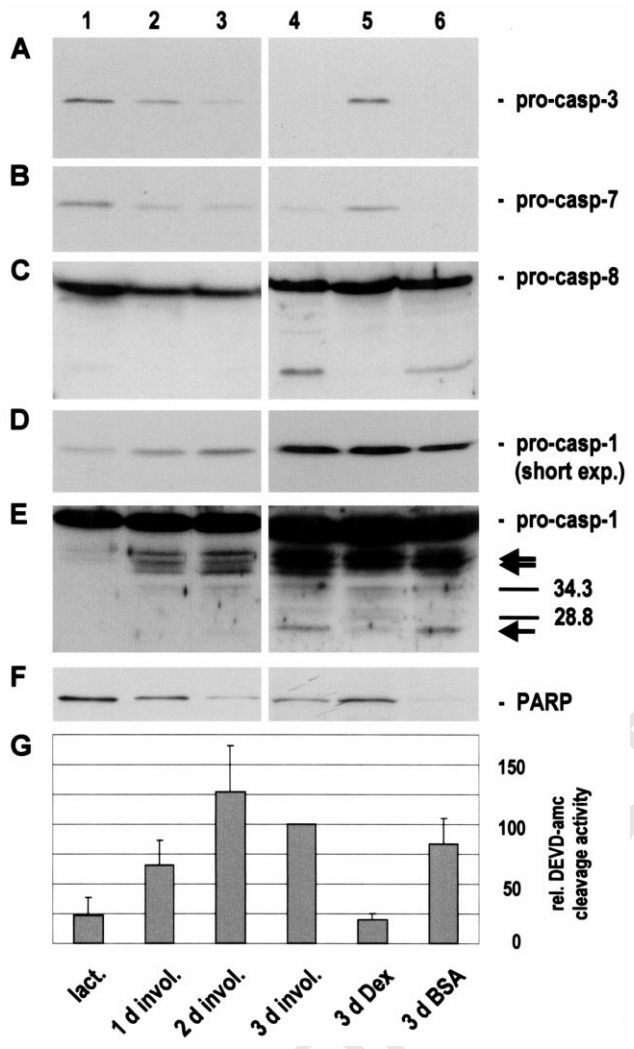

Fig. 2. Caspase processing and activity during normal mouse mammary gland involution and in dexamethasone-exposed glands. Extracts were prepared from mammary glands at lactation (lane 1), and at 1 (lane 2), 2 (lane 3) and 3 days of normal involution (lane 4), and 3 days after dexamethasone (lane 5) or control BSA-pellet implantation (lane 6). Western blot analyses were performed with cytoplasmic preparations using antibodies specific for caspase-3 (panel A), caspase-7 (panel B), caspase-8 (panel C), caspase-1 (short exposure panel D; and long exposure of the same blot panel E). PARP was determined in nuclear extracts (panel F). Arrows indicate processed forms of caspase-1. Caspase activity was analyzed by incubating nuclear extracts in the presence of DEVD–amc (panel G). Fluorescence was measured over a period of 50 min, and shown are the relative DEVD cleavage activities as arbitrary units of fluorescence. Values at 3 days of involution were set to 100%. Error bar, SD.

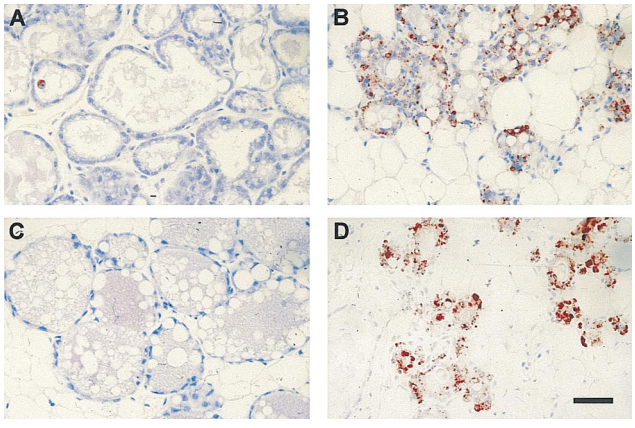

Fig. 3. Inhibition of caspase-3 by dexamethasone during involution. Sections derived from mammary glands at lactation (panel A), 3 days of involution (panel B), 3 days of involution in the presence of dexamethasone-release pellets (panel C) or 3 days of involution in the presence of BSA control pellets (panel D) were stained for activated caspase-3 with CM-1 antibody. The bar represents 100 μm.

weaning that were either implanted with dexamethasone containing pellets (panel C) or BSA control pellets (panel D). In sections of lactating glands, epithelial cells were negative for active caspases, with the exception of cells that fell into the alveolar lumen (panel A). In contrast, at 3 days of involution, alveoli in untreated glands and in BSA control glands were mostly collapsed and many epithelial cells stained positive for active caspases (panel B and D). Apoptotic nuclei were frequently detected, and terminal transferase-mediated labeling of free DNA ends (TUNEL staining) indicated that DNA fragmentation occurs in dying epithelial cells (data not shown; and Feng et al., 1995). In dexamethasone-treated glands, the alveolar structure remained intact and the glands were strongly engorged due to the accumulation of milk (panel C). However, only very few apoptotic nuclei were detected and TUNEL staining was negative (data not shown). Almost no epithelial cells stained positive with the CM-1 antibody. These results, together with the Western blot analyses shown in Fig. 2A, indicate that signal transduction pathways normally leading to caspase-3 processing were effectively blocked in vivo in the intact gland by dexamethasone.

2.4. Cytochrome c and caspase-9 during mammary involution in Bcl-2 transgenic glands

Bcl-2, a member of a larger family of pro- and anti-apoptotic proteins, is a potent inhibitor of apoptosis and mitochondrial release of cytochrome *c* in many systems (Reed, 1997, 1998). It was previously reported that Bcl-2 delayed mammary gland involution in transgenic mice expressing the human *bcl-2* gene under the control of the whey acidic protein (WAP) gene promoter in the mammary gland (Jäger et al., 1997). We analyzed the consequences of Bcl-2 expression on cytochrome *c* release, caspase activation and apoptosis in mammary glands of C57BL/6 mice at lactation and at 3 days of involution. Transgenic Bcl-2 protein was expressed at lactation (Fig. 4A, lane 3) and 3 days of involution (lane 4), whereas no signal was detected in wildtype animals (lanes 1 and 2). Interestingly, the Bcl-2 protein migrated as a doublet in extracts derived from glands at lactation (lane 3). During involution, the lower band was reduced (lane 4). At day 3 of involution, apoptosis was clearly inhibited in Bcl-2 overexpressing glands as compared with glands of wildtype animals (data not shown; and Jäger et al., 1997). Mitochondria-enriched fractions were prepared from wildtype and Bcl-2 transgenic mammary glands at lactation and at 3 days of involution. The amount of mitochondrial cytochrome *c* that was released at 3 days of involution was reduced in Bcl-2 transgenic glands (panel B, lane 4) as compared with wildtype control glands (lane 2). COX IV was analyzed to normalize for mitochondrial proteins (panel C, lanes 1–4). Caspase-9 was analyzed in cytoplasmic fractions of lactating and involuting mammary tissue. Caspase-9 processing was markedly inhibited in Bcl-2 transgenic glands at 3 days of involution (panel D, lane 4) as compared with wildtype glands (lane 2). Together, these results suggest that Bcl-2 overexpression delays apoptosis during mammary gland involution by reducing the mitochondrial efflux of cytochrome *c* and subsequent impairment of pro-caspase-9 processing.

2.5. Caspase processing and activity during involution in Bcl-2 transgenic glands

We further analyzed caspase-associated events that are expected to occur downstream of cytochrome *c* release and pro-caspase-9 processing in wildtype and Bcl-2 transgenic mammary glands during involution. Fig. 5 shows that the processing of pro-caspase-3 (panel A), pro-caspase-7 (panel B) and possibly pro-caspase-8 (panel C) were inhibited at 3 days of involution in Bcl-2 transgenic glands (lane 4) as compared with wildtype animals (lane 2). The levels of pro-caspase expression at lactation were comparable in wildtype (lane 1) and Bcl-2 transgenic glands (lane 3). Interestingly, the accumulation of caspase-1 during involution was similar in wildtype (panel D, lanes 1 and 2) and transgenic glands (lanes 3 and 4). In order to monitor caspase

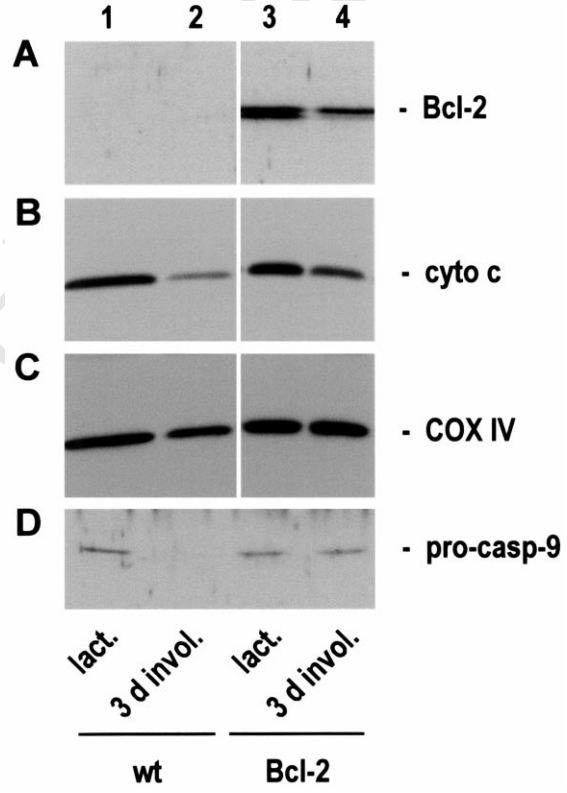

Fig. 4. Cytochrome *c* release and processing of caspase-9 during mammary gland involution in wildtype and Bcl-2 transgenic animals. Mitochondrial and cytoplasmic fractions were prepared from wildtype (lanes 1 and 2) and Bcl-2 transgenic mammary glands (lanes 3 and 4) at lactation (lanes 1 and 3) and at 3 days of involution (lanes 2 and 4). Western blot analyses were performed with cytoplasmic (panels A and D) and mitochondria-enriched fractions (panels B and C) using antibodies specific for Bcl-2 (panel A), cytochrome *c* (cyto c; panel B), COX IV (panel C), and caspase-9 (panel D).

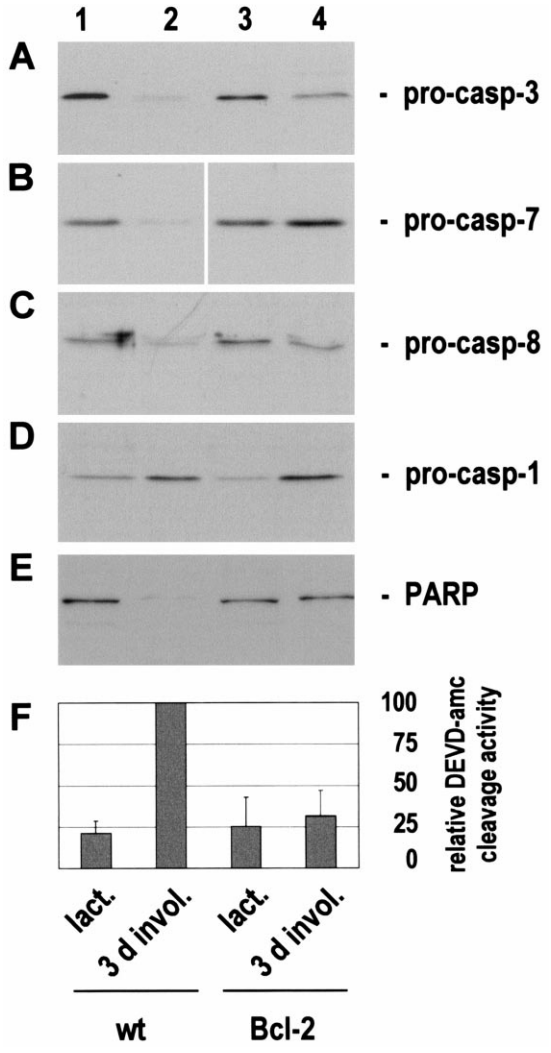

Fig. 5. Caspase processing and activity during mammary gland involution in wildtype and Bcl-2 transgenic animals. Extracts were prepared from wildtype (lanes 1 and 2) and Bcl-2 transgenic mammary glands (lanes 3 and 4) at lactation (lanes 1 and 3) and at 3 days of involution (lanes 2 and 4). Western blot analyses were performed with cytoplasmic extracts using antibodies specific for caspase-3 (panel A), caspase-7 (panel B), caspase-8 (panel C), caspase-1 (panel D). PARP was determined in nuclear extracts (panel E). Caspase activity was analyzed by incubating nuclear extracts in the presence of DEVD–amc (panel F). Fluorescence was measured over a period of 50 min and shown are the relative DEVD cleavage activities. Values at 3 days of involution were set to 100%. Error bar, SD.

activity, cleavage of nuclear PARP was analyzed (panel E). In wildtype glands, PARP was clearly reduced at day 3 of involution (lane 2). Similar to MORO mice, the expected p85 PARP fragment was not detected in extracts derived from involuting C57BL/6 mammary glands. The levels of PARP expression were similar at lactation (lane 3) and at 3 days after weaning (lane 4) in Bcl-2 transgenic glands, suggesting that caspase activity was impaired by overexpression of Bcl-2 during involution. This issue was further explored by monitoring caspase activity in a DEVD–amc cleavage assay. In wildtype animals, DEVD–amc cleavage activity was low in extracts prepared from glands at lactation (panel F, lane 1) and elevated at 3 days of involution (lanes 2). Bcl-2 overexpression resulted in an almost complete inhibition of caspase activation at 3 days of involution (lane 4).

The CM-1 antibody was used to analyze, in situ, the presence of active caspase-3 in wildtype and Bcl-2 transgenic glands. Mammary sections were stained with the CM-1 antibody, and Fig. 6 shows that caspase-3 activation is much reduced in Bcl-2 transgenic glands at day 3 of involution (panel D) as compared with wildtype control animals (panel B). Almost no signals were detected in lactating glands of wildtype (panel A) or transgenic animals (panel C). These results indicate that apoptosis of mammary epithelial cells during involution strongly correlates with the presence of active caspases.

3. Discussion

In this study, we investigated the release of cytochrome *c* as a possible trigger of caspase activation in vivo in the involuting mouse mammary gland (see also Fig. 7). Overexpression of Bcl-2 in the mammary gland results in an inhibition of cytochrome *c* release and subsequent processing of caspases, including caspase-1, -3, -7, -8 and -9. Inhibition of involution by implantation of dexamethasone-release pellets into glands similarly impairs caspase processing and apoptosis, but not the release of cytochrome *c* from mitochondria.

Possible triggers of mammary gland involution and epithelial cell apoptosis are systemic changes of lactogenic hormones and the local accumulation of milk, resulting in an engorgement of the gland and prominent shape change of epithelial cells (Topper and Freeman, 1980; Marti et al., 1997, 1999b). How these extracellular signals are biochemically coordinated and converted into an apoptotic response of the epithelium is presently unknown. An attractive

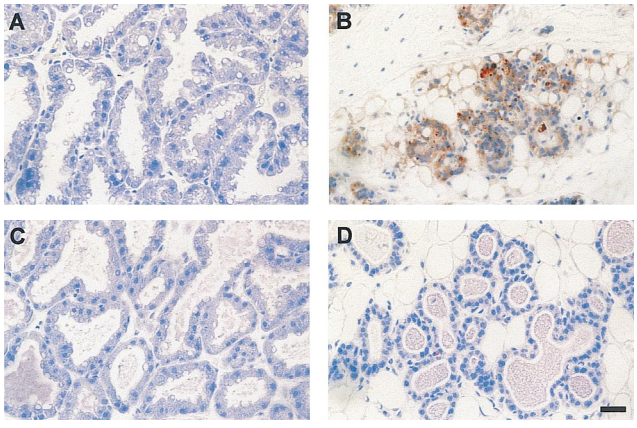

Fig. 6. Inhibition of caspase-3-like activity during involution by Bcl-2. Sections derived from wildtype (panels A and B) and Bcl-2 transgenic mammary glands (panels C and D) at lactation (panels A and C) or 3 days of involution (panels B and D) were stained for active caspases with CM-1 antibody. The bar represents 50 μm.

hypothesis predicts that local changes in hormone levels and epithelial cell shape changes influence the epithelial cell/ECM interactions. Streuli and co-workers showed in cultured mammary epithelial cells, that such changes result in a translocation of cytosolic Bax into mitochondrial membranes, resulting in a release of cytochrome *c*, activation of caspases and apoptosis (Gilmore et al., 2000).

3.1. Cytochrome c release and caspase activity during mammary involution

Apoptosis of mammary epithelial cells in vivo is associated with a loss of mitochondria-derived cytochrome *c*. Most likely, released cytochrome *c* associates in the cytosol with Apaf-1, forming the apoptosome that mediates the conversion of pro-caspase-9 into the processed, and most likely, active caspase-9 enzyme complex. This has been well-described during apoptosis of cells in culture, but to the best of our knowledge, it has not been conclusively shown during developmentally controlled programmed cell death in vivo. Due to the fact that cytochrome c gene-knockout animals do not survive, it will be difficult to ultimately prove whether the cytochrome *c* release is an initial and central caspase-activating event or the consequence of a caspase activation cascade, e.g. involving caspase-1 or caspase-8, initiated by death signals elsewhere in cells. However, in both cases, the loss of mitochondrial cytochrome *c* ensures the breakdown of the cellular metabolism and the irreversible execution of mammary epithelial cell death.

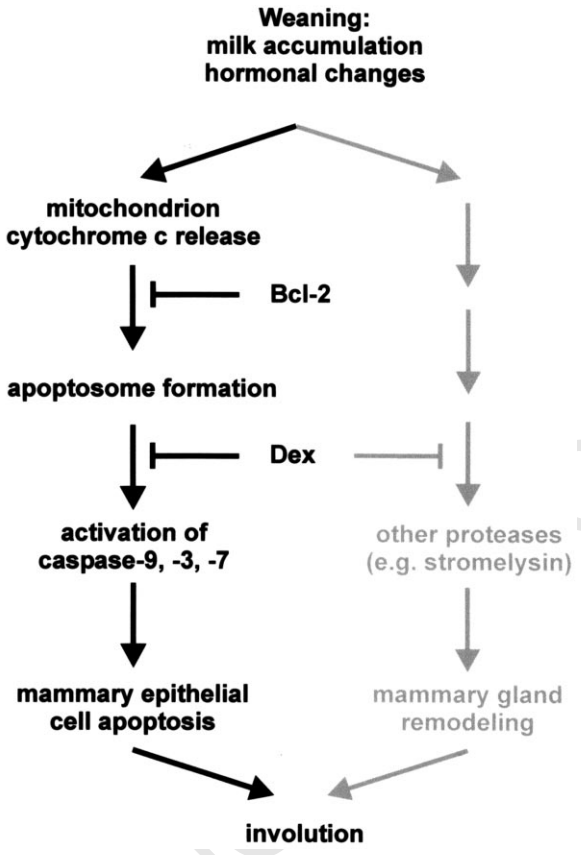

Fig. 7. Schematic diagram summarizing caspase-activating events and tissue remodeling during involution. After weaning, milk accumulation and hormonal changes result in the release of mitochondrial factors such as cytochrome *c*, activation caspases and subsequent epithelial cell apoptosis. These events are paralleled by processes leading to tissue remodeling, such as induction of metalloproteinase expression. Whereas Bcl-2 inhibits apoptosis at the level of mitochondria, dexamethasone inhibits caspase activation downstream of cytochrome *c* release, as well as tissue remodeling.

Our data suggest an involvement of caspase-9 during mammary epithelial cell death. Cleaved caspase-9 subunits become detectable at day 2 of involution, indicating an activation of caspase-9, most likely due to the accumulation of cytochrome *c* and the formation of an apoptosome in the cytosol. The accumulation of processed caspase-9 is paralleled by a transient increase of caspase-9 and caspase-3-like activity, as monitored by an induction of LEHD–afc and a DEVD–amc cleavage activity, peaking at day 2 of involution. In C57BL/6 mice, processed caspase-9 was not detectable. This may be due to the fact that apoptosis occurs in a less coordinated manner in this mouse strain as compared with MORO mice where the involution process is essentially terminated within a period of about 7 days.

Western blot analyses, immunohistochemical data and DEVD-assays suggest a role for caspase-3 during mammary epithelial cell death. Signals for activated caspase-3 can only be detected in the involuting epithelium and inhibition of involution and apoptosis either with dexamethasone or Bcl-2 overexpression results in a strong reduction of caspase-3 processing and activity in the gland.

Caspase-1 is, so far, the only caspase known to accumulate during involution. An induction of caspase-1 at the mRNA level in mammary epithelial cells during cell death was previously reported by Bissell and co-workers (Boudreau et al., 1995; Lund et al., 1996). Here, we show that caspase-1 also accumulates at the protein level during involution. Induction of caspase-1 was more pronounced in MORO mice as compared with C57BL/6 mice, most likely reflecting a strain-specific variation. Caspase-1 processing is an early event, already detectable at 1 day of involution. This is remarkable in the sense that caspase-1 was shown to have the ability to induce mitochondrial changes in certain cell types (Susin et al., 1997). Therefore, activation of caspase-1 may contribute to the loss of mitochondrial cytochrome *c* during mammary gland involution.

PARP represents a well-characterized caspase substrate. Our results indicate that PARP is cleaved during involution, however, we have not been able to detect the p85 kDa fragment in extracts derived from involuting mammary glands. The reason may be that epithelial cell apoptosis in the involuting mammary gland is a complex process involving phagocytosis of dying cells and tissue remodeling. Apoptosis in the involuting mammary gland occurs over a period of several days and is therefore much less synchronized than in cells in culture. Furthermore, many epithelial

cells are phagocytosed very early in vivo by neighboring epithelial cells or professional phagocytes (unpublished observation) and cellular fragments are removed to completion.

3.2. Dexamethasone inhibits caspase activity but not cytochrome c release

Glucocorticoids may act as survival factors of mammary epithelial cells and the administration of dexamethasone potently inhibits involution and apoptosis-associated induction of DNA fragmentation and the induction of tissue remodeling-associated proteases, such as stromelysin (Feng et al., 1995). The induction of other markers of involution such as activation of protein kinase A-like kinases and induction of AP1 (Fos/Jun) transcription factors are not inhibited in the presence of dexamethasone (Feng et al., 1995). Here, we show that dexamethasone potently inhibits caspase processing and DEVD cleavage activity. However, dexamethasone does not prevent the accumulation and partial processing of caspase-1 protein or the release of cytochrome *c* from mitochondria. Dexamethasone and possibly other glucocorticoids may therefore block the apoptotic pathway of mammary epithelial cells downstream or independent of mitochondria. Our results further demonstrate that the release of mitochondrial cytochrome *c* occurs independently of the activity of downstream executioner caspases, such as caspase-3 and -7. The fact that dexamethasone also inhibits caspase-8 processing indicates that caspase-8 activity may not be needed for cytochrome *c* release in dying mammary epithelial cells. Together, these results strongly suggest that cytochrome *c* release is an early event during apoptosis of mammary epithelial cells in the involuting gland and not merely the consequence of the activity of executioner caspases.

3.3. Bcl-2 inhibits cytochrome c release and caspases during mammary gland involution

To further characterize the role of cytochrome *c* in the apoptotic pathway during involution, transgenic animals were used that overexpress Bcl-2 in the mammary gland. It was previously shown that apoptosis is inhibited in Bcl-2 transgenic glands up to day 3 of involution, correlating with the expression of the transgene (Jäger et al., 1997). At later stages, involution also occurs in Bcl-2 transgenic animals, most likely due to inactivation of transgene expression in the gland. It is well-established that Bcl-2 controls the release of mitochondrial cytochrome *c* (Green and Reed, 1998) and it is very likely that Bcl-2 overexpression in the gland acts primarily by preventing cytochrome *c* release during involution in vivo. As expected, our studies reveal that inhibition of apoptosis by Bcl-2 is associated with a partial inhibition of cytochrome *c* release from mitochondria. The fact that caspase-9, -3 and -7 are not or only marginally processed and that caspase activity is impaired in transgenic glands corroborates the central role of cytochrome *c*. Interestingly, induction of caspase-1 protein is not inhibited in the transgenic gland. Therefore, caspase-1 induction is a very early or Bcl-2 independent event in the coordinated process of mouse mammary epithelial cell death. Since caspase-1 expression is not an absolute requirement for mammary gland involution (Li et al., 1995), additional studies are needed to clarify the contribution of caspase-1 for mammary gland involution. Future studies now need to address the question of which caspases are essential for mammary gland involution and epithelial cell death. Mammary gland-specific overexpression of distinct caspase inhibitors may shed light on the functional role of individual caspases during mammary gland involution.

4. Experimental procedures

4.1. Animals and tissue preparation

Involution was induced in MORO, C57BL/6 and WAP–Bcl-2 transgenic C57BL/6 mice after 5–7 days of lactation by removing the litter. Mammary gland tissue was prepared from wildtype or transgenic animals at the indicated time points, fixed in freshly prepared 4% paraformaldehyde phosphate-buffered saline and embedded in paraffin. Sections, 4 μm thick, were stained with hematoxylin and eosin or used for immunohistochemistry. Part of the tissue was frozen in liquid nitrogen and stored at 70°C.

4.2. Preparation and implantation of Elvax release pellets

Dexamethasone (Sigma) was dissolved in 70% ethanol, mixed with BSA and lyophilized. Elvax (10%) dissolved in methylene chloride was added, and the mixture was again lyophilized. Pellets were cut to a size of approximately 1.0 mg. One pellet contained 0.5 mg BSA in combination with 60 μg steroid hormone. Control pellets contained 0.5 mg BSA. Lactating animals were anesthetized by intraperitoneal injection (50 μl/10 g body weight) of a mixture containing ketamin (Ketasol-100, 2.4 × diluted in PBS, Graeub AG, Bern, Switzerland) and xylazin (Rompun, 6.3 × diluted in PBS, Bayer AG, Leverkusen, Germany). Two Elvax pellets were implanted into one of the fourth inguinal mammary glands by cutting a small pocket with an iris scissors. Implanted and matched glands were analyzed at the indicated time points.

4.3. Immunohistochemistry

For immunohistochemistry, 4 μm thick sections were treated with 0.6% H_2O_2 in methanol for 30 min at room temperature to inactivate endogenous peroxidase. Nonspecific binding was blocked with normal goat serum (Kirkegaard & Perry, Gaithersburg, MD). Sections were incubated overnight at 4°C with rabbit polyclonal CM-1 antibody (dilution, 1:2000; a gift from A. Srinivasan) that recognizes active caspase-3-like caspases (Srinivasan et al.,

1998). Antigen–antibody complexes were detected with the dextran-based, biotin free horseradish peroxidase conjugate (EnVision™ + System; DAKO, Glostrup, Denmark) using 3-amino-9-ethylcarbazole as substrate.

4.4. Extract preparation and Western blot analysis

Frozen tissue (approximately 300 mg) was homogenized with a polytrone on ice in 600 μl solution A (10 mM HEPES (pH 7.9), 1.5 mM magnesium chloride, 10 mM potassium chloride, 1 mM β-mercaptoethanol, 1 mM DTT) in the presence of protease inhibitors (5 μg/ml leupeptin, 50 μg/ml aprotinin, 1 mM amino-hexanoic acid, 5 μg/ml antipain, 5 μg/ml pepstatin, 5 μg/ml chymostatin, 5 mM benzamidine, 0.125 mM PMSF). After incubation on ice for 10 min, extracts were vortexed for 10 s and centrifuged (Sigma 202-MK) at 3500 revs./min for 2 min at 4°C to pellet nuclei. The supernatant was centrifuged at 13 000 revs./min at 4°C for 30 min. The cleared supernatant was taken as the cytoplasmic extract and the pellet as the mitochondria-enriched fraction. The mitochondria-enriched fraction was directly dissolved in SDS-sample buffer and boiled. Nuclear pellets were incubated in 50 μl Solution C (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 5 μg/ml leupeptin, 50 μg/ml aprotinin, 1 mM amino-hexanoic acid, 5 μg/ml antipain, 5 μg/ml pepstatin, 5 μg/ml chymostatin, 5 mM benzamidine, 0.125 mM PMSF and 25% glycerol) on ice for 30 min and centrifuged at 15 000 × *g* for 30 min at 4°C (Sigma 202-MK). The supernatant was taken as nuclear extract. Protein concentrations in cytoplasmic and nuclear extracts were determined using the Bio-Rad protein assay. Extracts were stored at 70°C. For Western blot analysis, 30 μg cytoplasmic or nuclear extract or the corresponding amount of protein from the mitochondria-enriched fraction (as judged by Ponceau staining) was separated on a 12% SDS polyacrylamide gel. After transfer to nitrocellulose, proteins were detected with the corresponding antibodies using the Western blot chemiluminescence reagent plus, following the manufacturer's instructions (NEN, Life Science Products, Boston, MA). Non-specific binding was blocked with PBS, 5% milk and 0.2% Tween 20 prior to incubation with the first antibody. The following antibodies were used for Western blot analyses: mouse monoclonal anti-cytochrome *c* (7H8.2C12, Pharmingen, San Diego, CA), mouse monoclonal COX IV (20E8-C12, Molecular Probes, Eugene, OR), mouse monoclonal anti-PARP (C-2-10, Biomol, Plymouth Meeting, Philadelphia, PA), rabbit polyclonal anti-caspase-9 (Krajewski et al., 1999), rabbit polyclonal anti-human-Bcl-2 (a kind gift from J.C. Reed), rabbit polyclonal anti-caspase-9 (a kind gift from D. Green), mouse caspase-specific rabbit polyclonal anti-caspase-1, -3, -7, and -8 (generated in the laboratory of P. Vandenabeele).

4.5. Fluorometric caspase assays

Nuclear extracts (30 μg protein) were mixed with 32 μl caspase buffer (Promega, Madison, WI), 2 μl DMSO, 1 μl 1 M DTT, 1 μl synthetic DEVD–amc caspase-3 substrate (10 mM DEVD–amc stock solution in DMSO, Calbiochem, La Jolla, CA) and filled up to 100 μl with H_2O. The emitting fluorescence was kinetically measured at 30°C for 50 min with a Spectramax Gemini Fluorometer (Molecular Devices, Sunnyvale, CA). The relative cleavage was determined by calculating the slope of the accumulation of amc fluorochrome during the assay. For statistical analyses, at least three series of animals (lactation and 1–3 days of involution) were analyzed. In each series, caspase activity at 3 days of involution was set to 100% and the other values were calculated relative to 3 days of involution.

Acknowledgements

The authors wish to thank D. Green (La Jolla Institute for Allergy and immunology, La Jolla, CA) for providing anti-caspase-9 antibody; S. Krajewski (The Burnham Institute, La Jolla, CA) and J.C. Reed (The Burnham Institute, La Jolla, CA) for providing anti-caspase-9 and anti-Bcl-2 antibodies; and A. Srinivasan (IDUN, La Jolla, CA) for providing CM-1 antibody. This work was supported by the Swiss National Science Foundation, the Sandoz Foundation and the Foundation for Clinical, Experimental Cancer Research (Switzerland) and the IAP funding (Phase IV) from the Belgian Government (W. Declercq, P. Vandenabeele).

References

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., Yuan, J., 1996. Human ICE/CED-3 protease nomenclature. Cell 87, 171.

Boldin, M.P., Goncharov, T.M., Goltsev, Y.V., Wallach, D., 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85, 803–815.

Boudreau, N., Sympson, C.J., Werb, Z., Bissell, M.J., 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science 267, 891–893.

Cecconi, F., 1999. Apaf1 and the apoptotic machinery. Cell Death Differ. 6, 1087–1098.

Cecconi, F., Alvarez-Bolado, G., Meyer, B.I., Roth, K.A., Gruss, P., 1998. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell 94, 727–737.

Chapman, R.S., Lourenco, P.C., Tonner, E., Flint, D.J., Selbert, S., Takeda, K., Akira, S., Clarke, A.R., Watson, C.J., 1999. Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev. 13, 2604–2616.

Deveraux, Q.L., Reed, J.C., 1999. IAP family proteins – suppressors of apoptosis. Genes Dev. 13, 239–252.

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

Gilmore, A.P., Metcalfe, A.D., Romer, L.H., Streuli, C.H., 2000. Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. J. Cell Biol. 149, 431–446.

Green, D.R., Reed, J.C., 1998. Mitochondria and apoptosis. Science 281, 1309–1312.

Hakem, R., Hakem, A., Duncan, G.S., Henderson, J.T., Woo, M., Soengas, M.S., Elia, A., de la Pompa, J.L., Kagi, D., Khoo, W., Potter, J.,

Yoshida, R., Kaufman, S.A., Lowe, S.W., Penninger, J.M., Mak, T.W., 1998. Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell 94, 339–352.

Jäger, R., Herzer, U., Schenkel, J., Weiher, H., 1997. Overexpression of Bcl-2 inhibits alveolar cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice. Oncogene 15, 1787–1795.

Kerr, J.F., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239–257.

Kidd, V.J., 1998. Proteolytic activities that mediate apoptosis. Annu. Rev. Physiol. 60, 533–573.

Krajewski, S., Krajewska, M., Ellerby, L.M., Welsh, K., Xie, Z., Deveraux, Q.L., Salvesen, G.S., Bredesen, D.E., Rosenthal, R.E., Fiskum, G., Reed, J.C., 1999. Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc. Natl. Acad. Sci. USA 96, 5752–5757.

Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P., Flavell, R.A., 1998. Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. Cell 94, 325–337.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., et al., 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell 80, 401–411.

Li, M., Liu, X., Robinson, G., Bar-Peled, U., Wagner, K.U., Young, W.S., Hennighausen, L., Furth, P.A., 1997a. Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc. Natl. Acad. Sci. USA 94, 3425–3430.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X., 1997b. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479–489.

Li, K., Li, Y., Shelton, J.M., Richardson, J.A., Spencer, E., Chen, Z.J., Wang, X., Williams, R.S., 2000. Cytochrome *c* deficiency causes embryonic lethality and attenuates stress-induced apoptosis. Cell 101, 389–399.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 86, 147–157.

Lund, L.R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M.J., Dano, K., Werb, Z., 1996. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development 122, 181–193.

Marti, A., Jehn, B., Costello, E., Keon, N., Ke, G., Martin, F., Jaggi, R., 1994. Protein kinase A and AP-1 (c-Fos/JunD) are induced during apoptosis of mouse mammary epithelial cells. Oncogene 9, 1213–1223.

Marti, A., Feng, Z., Altermatt, H.J., Jaggi, R., 1997. Milk accumulation triggers apoptosis of mammary epithelial cells. Eur. J. Cell Biol. 73, 158–165.

Marti, A., Jaggi, R., Vallan, C., Ritter, P.M., Baltzer, A., Srinivasan, A., Dharmarajan, A.M., Friis, R.R., 1999a. Physiological apoptosis in hormone-dependent tissues: involvement of caspases. Cell Death Differ. 6, 1190–1200.

Marti, A., Lazar, H., Ritter, P., Jaggi, R., 1999b. Transcription factor activities and gene expression during mouse mammary gland involution. J. Mammary Gland Biol. Neoplasia 4, 145–152.

Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E., Dixit, V.M., 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85, 817–827.

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

Porter, A.G., Jänicke, R.U., 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 6, 99–104.

Reed, J.C., 1997. Double identity for proteins of the Bcl-2 family. Nature 387, 773–776.

Reed, J.C., 1998. Bcl-2 family proteins. Oncogene 17, 3225–3236.

Rosen, A., Casciola-Rosen, L., 1997. Macromolecular substrates for the ICE-like proteases during apoptosis. J. Cell. Biochem. 64, 50–54.

Salvesen, G.S., Dixit, V.M., 1997. Caspases: intracellular signaling by proteolysis. Cell 91, 443–446.

Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., Wang, H.G., Reed, J.C., Nicholson, D.W., Alnemri, E.S., Green, D.R., Martin, S.J., 1999. Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J. Cell Biol. 144, 281–292.

Srinivasan, A., Roth, K.A., Sayers, R.O., Shindler, K.S., Wong, A.M., Fritz, L.C., Tomaselli, K.J., 1998. In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. Cell Death Differ. 5, 1004–1016.

Strange, R., Li, F., Saurer, S., Burkhardt, A., Friis, R.R., 1992. Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development 115, 49–58.

Susin, S.A., Zamzami, N., Castedo, M., Daugas, E., Wang, H.G., Geley, S., Fassy, F., Reed, J.C., Kroemer, G., 1997. The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. J. Exp. Med. 186, 25–37.

Talhouk, R.S., Bissell, M.J., Werb, Z., 1992. Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. J. Cell Biol. 118, 1271–1282.

Thornberry, N.A., Lazebnik, Y., 1998. Caspases: enemies within. Science 281, 1312–1316.

Topper, Y.J., Freeman, C.S., 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. 60, 1049–1106.

Tschopp, J., Irmler, M., Thome, M., 1998. Inhibition of fas death signals by FLIPs. Curr. Opin. Immunol. 10, 552–558.

Vaux, D.L., 1993. Toward an understanding of the molecular mechanisms of physiological cell death. Proc. Natl. Acad. Sci. USA 90, 786–789.

Vaux, D.L., Strasser, A., 1996. The molecular biology of apoptosis. Proc. Natl. Acad. Sci. USA 93, 2239–2244.

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

Wang, S., Miura, M., Jung, Y.K., Zhu, H., Li, E., Yuan, J., 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell 92, 501–509.

Wyllie, A.H., 1995. The genetic regulation of apoptosis. Curr. Opin. Genet. Dev. 5, 97–104.

Wyllie, A.H., Kerr, J.F., Currie, A.R., 1980. Cell death: the significance of apoptosis. Int. Rev. Cytol. 68, 251–306.

Yoshida, H., Kong, Y.Y., Yoshida, R., Elia, A.J., Hakem, A., Hakem, R., Penninger, J.M., Mak, T.W., 1998. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 94, 739–750.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., Wang, X., 1997. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome *c*-dependent activation of caspase-3. Cell 90, 405–413.