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Research Article

Apoptotic cell death in the lactating mammary gland is enhanced by a folding variant of α-lactalbumin

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Abstract. Apoptosis is essential to eliminate secretory epithelial cells during the involution of the mammary gland. The environmental regulation of this process is however, poorly understood. This study tested the effect of HAMLET (human α-lactalbumin made lethal to tumor cells) on mammary cells. Plastic pellets containing HAMLET were implanted into the fourth inguinal mammary gland of lactating mice for 3 days. Exposure of mammary tissue to HAMLET resulted in morphological changes typical for apoptosis and in a stimulation of caspase-3 activity in alveolar epithelial cells near the HAMLET pellets but not more distant to the pellet or in contralateral glands. The effect was specific for HAMLET and no effects were observed when mammary glands were exposed to native α-lactalbumin or fatty acid alone. HAMLET also induced cell death in vitro in a mouse mammary epithelial cell line. The results suggest that HAMLET can mediate apoptotic cell death in mammary gland tissue.

Key words. Apoptosis; α-lactalbumin; breast cancer; HAMLET; caspase; involution; protein folding.

The mammary gland develops during embryogenesis to a mammary Anlage consisting of epithelium embedded in adipocytes. A first burst of epithelial proliferation occurs during puberty when an extensive ductal network is formed. Pregnancy stimulates massive epithelial cell proliferation and the formation of extended lobulo-alveolar structures which terminally differentiate toward the end of pregnancy. Continuous milk production by alveolar cells is maintained during lactation via the suckling stimulus exerted by the pups and elevated levels of glucocorticoids and prolactin [1, 2]. Weaning causes involution of the mammary gland, and the majority of secretory epithelial cells are removed by apoptotic cell death. The remaining structures are reorganized until a state resembling the virgin gland is reached [3, 4]. Apoptosis of mammary epithelial cells is regulated at the molecular level by cytochrome c release, proteolytic processing and activation of several caspases, e.g. caspase-1, -3, -7, -8 and -9 [5, 6].

Involution occurs through programmed cell death in the mammary gland, but the molecular events which initiate or regulate apoptosis of secretory epithelial cells remain unclear. Hormonal withdrawal, loss of mechanic stimulation and resulting local tissue responses contribute and overlap to safeguard this program [2, 7, 8]. For example, expression of transforming growth factor (TGF)-β3 is strongly induced in alveolar epithelial cells after weaning before apoptosis is initiated [9]. The importance of TGF-β3 on mammary epithelial cells was further documented in transgenic mice by showing that overexpression induced apoptosis during lactation while apoptosis was reduced but not fully absent in TGF-β3 knockout animals [10]. Apoptosis in the mammary gland requires de novo gene expression in mammary epithelial cells and imme-

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diate early genes like fos and jun, members of transcription factor AP-1, are up-regulated at the RNA and protein level during involution leading to the expression of AP-1-dependent genes [11]. Moreover, ATF4, another basic leucine zipper-containing activating transcription factor which can form heterodimers with Fos and Jun members, seems to be important. Overexpression of ATF4 in transgenic animals was associated with up-regulation of $p21^{WAF1}$ and $p27^{Kip1}$, with a stimulation of phosphorylation and nuclear translocation of Stat3 and an acceleration of involution [12]. In fact, Stat3 is also activated during normal involution and it may be a driving force during apoptosis of mammary epithelial cells [13]. More recently, leukemia inhibitory factor (LIF), which is induced during early involution, was shown to stimulate Stat3 phosphorylation [14].

HAMLET is a protein-lipid complex that induces cell death selectively in tumor cells and immature cells, but spares healthy cells. The protein component of HAMLET is α-lactalbumin (α-LA), the most abundant protein in human milk. α-LA is a globular, 14-kDa protein consisting of four α helices, a triple-stranded anti-parallel β sheet and a high-affinity calcium-binding site [15] that defines the native conformation of the protein [16]. In HAMLET, α-LA is partially unfolded and thus the conversion of α-LA to HAMLET involves the removal of calcium. The lipid component in HAMLET is the C18:1, 9cis fatty acid (oleic acid) which stabilizes the complex [17]. HAMLET shows broad activity against many different tumor cell lines in vitro, embryonal cells are moderately sensitive, while healthy, differentiated cells are resistant to the apoptosis-inducing effect. HAMLET acts by gaining entry into the tumor cells and redistributing to the nucleus, where it binds to histones and disrupts chromatin structures [18]. In the cytoplasm, HAMLET interacts with mitochondria, causing the release of cytochrome c followed by caspase activation [19].

Intuitively, molecules in human milk may influence the involution of the mammary gland, but such mechanisms have not been described. This study investigated whether HAMLET may play a physiological role in the mammary gland, as this is the major site of α-LA synthesis. Using an in vivo model of mammary gland involution, we show that HAMLET enhances apoptosis in alveolar epithelial cells upon weaning. We conclude that HAMLET may act as a homeostatic factor during the involution of the mammary gland.

Materials and methods

Preparation of HAMLET and HAMLET-releasing ELVAX pellets

HAMLET was prepared from human milk as described previously [20, 21]. HAMLET-releasing pellets were generated by mixing 40 mg of lyophilized HAMLET or α-LA with 160 µl of methylene chloride containing 10% ELVAX. The mixture was lyophilized and the resulting large pellet was cut into pieces containing approx. 1, 2.5 or 5 mg of HAMLET. Cytotoxic HAMLET was formed by incubating α-LA with oleic acid. Oleic acid-containing pellets were prepared by mixing the fatty acid with 10% ELVAX in methylene chloride and cutting the pellet into smaller pieces after lyophilization. Pellets were stored at –20 °C until use.

Animals, surgery and tissue preparation

Normal involution was induced in NMRI mice after 5–7 days of lactation by removing the litter. Animals were sacrificed at 3 days of involution and the fourth inguinal mammary glands were removed, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin.

For pellet implantation experiments, lactating mice were anesthetized by injecting 50 µl/10 g of xylazine (0.5% Rompun; Bayer, Leverkusen, Germany) and ketamine (40 mg/ml Ketasol-100; Dr. E. Gräub AG, Bern, Switzerland). The skin was opened near the nipple of the fourth inguinal gland by a short snip about 5 mm in length and a single pellet containing 1, 2.5 or 5 mg HAMLET, 5 mg α-LA, oleic acid or pure ELVAX was placed between the surface of the glandular structure and the connective tissue which spans the gland. Care was taken not to disturb ductal structures of the gland. Lactation was continued by returning the animals to their litter. Animals were sacrificed 3 days later. The piece of gland containing the implanted pellet was fixed with 4% PFA and embedded along with control material derived from more distant areas of the same gland and tissue from the contralateral gland. A total of five animals were implanted with pellets containing 1 mg of HAMLET, three animals with pellets containing 2.5 mg and three animals with pellets containing 5 mg of HAMLET. As controls, six animals were implanted with pellets containing 5 mg α-LA, three animals with oleic acid-containing pellets and five animals with 'empty' ELVAX pellets. Animal experiments were carried out according to the 'Ethical principles and guidelines for scientific experiments on animals'.

Cell culture, DAPI staining and preparation of cells for immunocytochemistry

31D cells are a non-tumorigenic mouse mammary epithelial cell line derived from the mammary gland of mid-pregnant Balb/c mice [22]. Cells were grown in DMEM containing 10% fetal calf serum, 10 ng/ml epidermal growth factor (Sigma, Buchs, Switzerland) and 5 µg/ml insulin (Sigma) in an atmosphere containing 5% CO_2 at 37 °C. Before treatment, cells were washed twice with phosphate-buffered saline (PBS) and incubated in serum-free medium. HAMLET was freshly dissolved in DMEM at 2 mg/ml and added to the cells. After incubating for

24 h, the cells were trypsinized, washed with cold PBS, fixed in 4% PFA for 10 min and stained with DAPI (Roche Diagnostics, Basel, Switzerland) for 20 min at room temperature. The percentage of cells with condensed chromatin was determined from at least 300 nuclei. Conditions (0 and 2 mg/ml HAMLET, 2 mg/ml α-LA, 10 µg/ml cisplatin) were tested in at least three independent experiments.

For immunocytochemistry, approx. 50,000 native cells (floating cells and trypsinized cells pooled) were plated by cytospin (Cytospin2 centrifuge; Shandon, DAKO Diagnostics, Zug, Switzerland), fixed with 4% PFA for 10 min and washed twice with cold PBS. Cells were stored in 70% ethanol until immunocytochemistry or the TUNEL reaction were performed.

Immunohistochemistry

Fixed cells or 6-µm-thick sections were treated with 0.6% H_2O_2 in methanol for 30 min to block endogenous peroxidases, incubated with normal goat serum for 30 min (Kirkegaard and Perry, Socochim, Lausanne, Switzerland) and then overnight with anti-active caspase-3 (CM1) antibody [23] (PAbCM1, rabbit polyclonal; Pharmingen, Becton Dickinson, Basel, Switzerland) at a dilution of 1:2000 in normal goat serum at 4 °C. Detection of antigen-antibody complexes was performed with AEC (Envision System; DAKO, Glostrup, Denmark), cells or sections were briefly counterstained with hematoxylin and covered with Aquatex (Merck, Grogg-Chemie, Stettlen-Deisswil, Switzerland). Pictures were taken with a DXM 1200 Nikon digital camera on a Nikon Eclipse 800 microscope.

TUNEL reaction

Fixed cells or 6-µm-thick sections were incubated with 1.2 mg/ml proteinase K (Roche) for 10 min at 21 °C and then with terminal transferase (Roche) and digoxigenin-11-dUTP (DIG; Roche) at 37 °C for 40 min. DIG was detected with anti-DIG-AP (Roche) at 1:2000 followed by a reaction with NBT (Roche) and BCIP (Roche). After brief counterstaining with hematoxylin, cells or sections were covered with Aquatex (Merck) and photographed.

Results

Involution of the mouse mammary gland in response to weaning

Mammary gland involution was induced in mice by removing the litter after 5–7 days of lactation, starting within 1 or 2 days and reaching a maximum after about 3 days. An initial engorgement is provoked by the accumulation of milk in alveolar structures of the gland, but after about 3 days post-weaning, milk is resorbed, alveolar structures collapse and secretory epithelial cells die by an apoptosis-like mechanism which was characterized by morphology, caspase-3 activation and TUNEL staining (data not shown). Apoptotic cells are rapidly phagocytosed by lymphocytes or neighboring cells and disappear from the gland. As a consequence, a relatively small fraction of dying cells is observed at any stage of involution (less than 3%). A similar sequence of events was previously shown in other mouse strains [4, 11, 24].

Processing of procaspase-3 and fragmentation of nuclear DNA in lactating mammary glands after exposure to HAMLET

The parameters of cell death and involution of the mammary gland were studied after exposure to HAMLET, using native α-LA or oleic acid as controls. ELVAX plastic pellets containing 5 mg HAMLET were implanted into the fourth inguinal mammary gland of lactating mice close to the prominent lymph node. Care was taken not to disrupt lobular structures of the gland since this would block milk removal from areas distal to the site of implantation. Animals were sacrificed 3 days after implantation and glands containing the pellet were removed as a whole. The pellet was localized visually and the area of the gland containing the pellet was fixed, embedded in paraffin and stained with hematoxylin and eosin. A collapse of alveolar structures (fig. 1A) and an appearance of apoptotic bodies (fig. 1A, inset) was observed in glands exposed to HAMLET but no signs of regression were observed more distant to the pellet (fig. 1B) or in contralateral glands (not shown). Glands exposed to α-LA (fig. 1C), oleic acid (fig. 1D) or to untreated ELVAX pellets (fig. 1E) were similar to normal glands during lactation.

Caspase-3 activation in response to HAMLET was examined with the CM1 antibody. Staining was observed in numerous alveolar epithelial cells in the immediate vicinity of the pellet (fig. 2A) but not in cells located more distant to the pellet (fig. 2B) or in contralateral glands (not shown). There was no evidence of caspase-3 activation after implantation of α-LA (fig. 2C), oleic acid (fig. 2D) or untreated ELVAX pellets (fig. 2E). A diffuse staining in the immediate vicinity of the implanted pellets was seen in some glands. This staining was most likely generated by stromal cells or infiltrating lymphocytes and reflects a local immune response after surgery.

Fragmentation of chromosomal DNA was studied by the TUNEL assay. TUNEL staining was observed in secretory epithelial cells exposed to pellets containing 5 mg HAMLET (fig. 3A) within a few hundred micrometers of the pellet. More distant to the pellet, no TUNEL-positive cells were observed (data not shown). The effect was concentration dependent, as shown by a weaker response with pellets containing 1 mg HAMLET (fig. 3B). No reaction was observed in tissues exposed to α-LA (fig. 3C), oleic acid (fig. 3D) or untreated pellets (fig. 3E). Again,

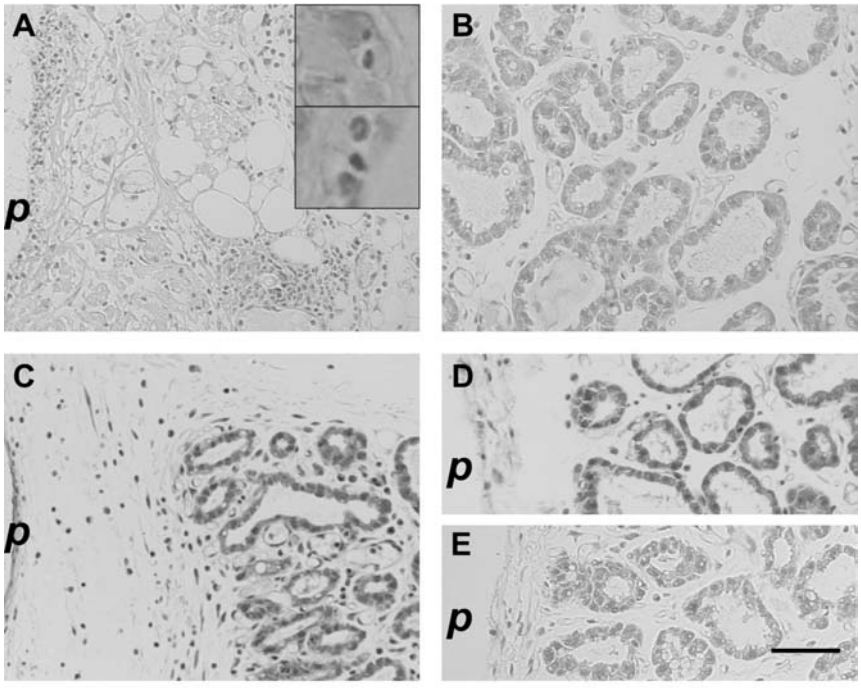

Figure 1. Morphological changes in luminal epithelial cells in the vicinity of plastic pellets. ELVAX pellets containing 5 mg HAMLET (*A*, *B*), 5 mg inactive α-LA (*C*), oleic acid (*D*) or an 'empty' pellet (*E*) were implanted into the fourth inguinal mammary gland of lactating NMRI mice 5–7 days after parturition. Lactation was continued and mammary glands were removed 3 days later and pellet-containing pieces of the gland were fixed, embedded and cut into 6-µm-thick sections. Sections were stained with hematoxylin and eosin. *B* represents a region of the same section as shown in *A* but located more distant to the pellet. Each condition was tested in several animals and shown are representative pictures (see Material and methods for more details). The position of the pellet is indicated (p). The bar represents 50 µm. Insets in *A* show apoptotic cells at higher magnification.

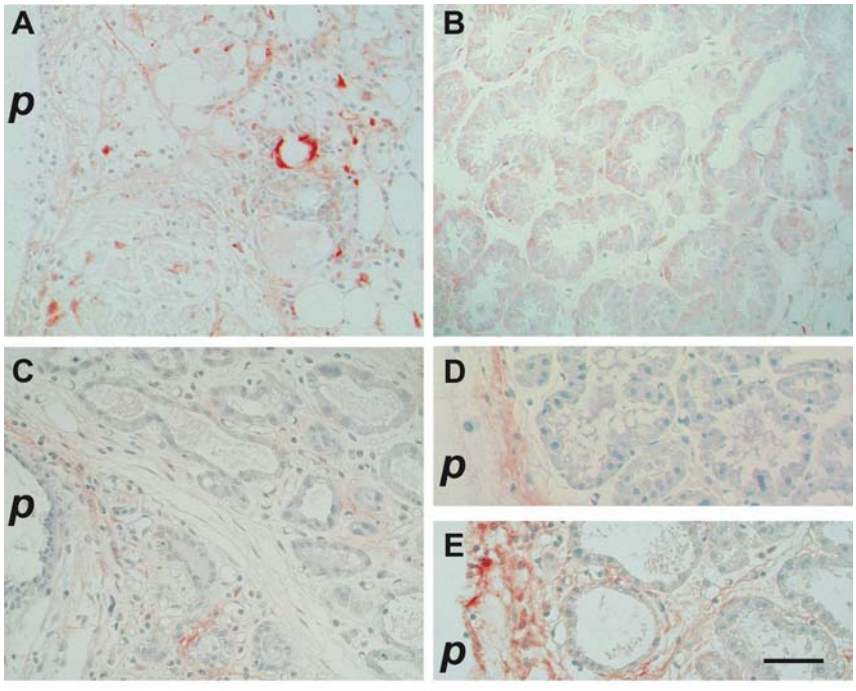

Figure 2. Evaluation of processing of procaspase-3 in response to HAMLET-releasing pellets. ELVAX pellets containing 5 mg of HAMLET (*A*, *B*), 5 mg of inactive α-LA (*C*), oleic acid (*D*) or an 'empty' pellet (*E*) were implanted into the fourth inguinal mammary gland of lactating NMRI mice 5–7 days after parturition. Lactation was continued and mammary glands were removed 3 days later and pellet-containing pieces of each gland were fixed, embedded and cut into 6-µm-thick sections and processed for CM1 staining. *B* represents a region of the same section as in *A* but located more distant to the pellet. Several animals were tested for each experimental condition and similar results were obtained (see Material and methods for more details). The bar represents 50 µm.

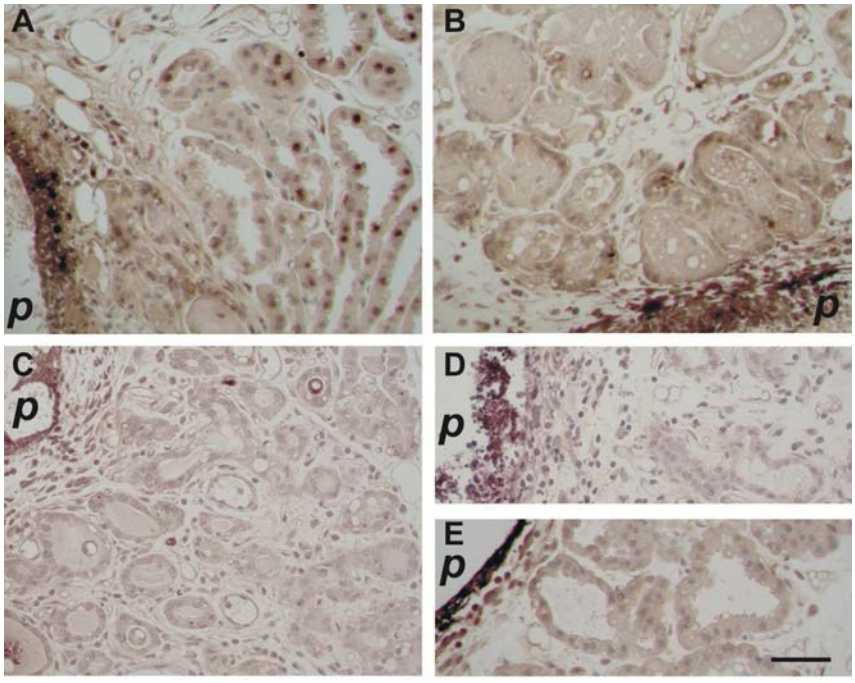

Figure 3. Fragmentation of chromosomal DNA in the mammary gland after exposure to HAMLET. Pellets containing 5 (*A*) or 1 (*B*) mg HAMLET, 5 mg of α-LA (*C*), oleic acid (*D*) or an 'empty' pellet (*E*) were implanted into the fourth inguinal mammary gland of lactating animals. Pellet-containing areas of the gland were removed 3 days later, fixed and embedded. Six-µm-thick sections were processed for TUNEL staining as described in Materials and methods. Several animals were tested for each experimental condition and similar results were obtained (see Materials and methods for details). The bar represents 50 µm.

a local, most likely immunological, reaction occurred in the immediate proximity of HAMLET-releasing pellets and some of these infiltrating lymphocytes or stromal cells were TUNEL positive. This non-specific reaction was independent of the type of pellet implanted.

These results suggest that HAMLET induces a local regression of alveolar structures, an induction of caspase-3-like activity and a fragmentation of chromosomal DNA. The same changes occur in the post-lactational mammary gland during normal involution. The frequency of apoptotic figures in the vicinity of HAMLET-containing pellets was similar to that in normal involution (data not shown).

Our observations strongly suggest that HAMLET stimulates a regression of epithelial structures and an apoptotic cell death of secretory epithelial cells in the lactating mammary gland in vivo.

HAMLET induces apoptotic cell death in non-transformed mouse mammary epithelial cells

The 31D cell line was originally cloned from IM-2, a mixed cell culture of epithelial and mesenchymal cells derived from the fourth inguinal gland of mid-pregnant mice [22]. The 31D cells have an epithelial phenotype and have retained some of their original physiological properties: e.g. they can be stimulated with lactogenic hormones (prolactin, glucocorticoids, insulin) to synthesize milk proteins like β-casein [25]. In this study, the effect of HAMLET on chromatin was examined. Exponentially growing cells were treated with HAMLET for 24 h and condensed chromatin was visualized by DAPI staining. Cells were treated with increasing concentrations of HAMLET or with cisplatin (CP) as a positive control. HAMLET caused a dose-dependent response and at 2 mg/ml, almost 20% of the cells became apoptotic within 24 h of treatment. In comparison, about 42% of all cells died after 24 h of treatment with 10 µg/ml cisplatin (fig. 4A). The 31D cells were insensitive to native α-LA, and confluent cell layers did not respond to HAMLET or cisplatin (fig. 4A).

Caspase-3 activation was quantified as the fraction of CM1-positive cells. HAMLET caused a 4.5-fold and CP a 10-fold increase in caspase-3 reactivity compared to untreated cells (fig. 4B, panels a, b and c). HAMLET and CP were also shown to stimulate nuclear DNA fragmentation by 3- and 8-fold, respectively (fig. 4B, panels d, e and f). Spontaneous cell death in untreated control cells was rare (0.7 ± 0.2%; fig. 4A) and cells were negative after CM1 staining (fig. 4B, panel c) or TUNEL labeling (fig. 4B, panel f). The fact that confluent cells are highly resistant to cell death implies that apoptosis by HAMLET (and CP) is dependent on factors that are only present or active in proliferating cells and not in confluent cells.

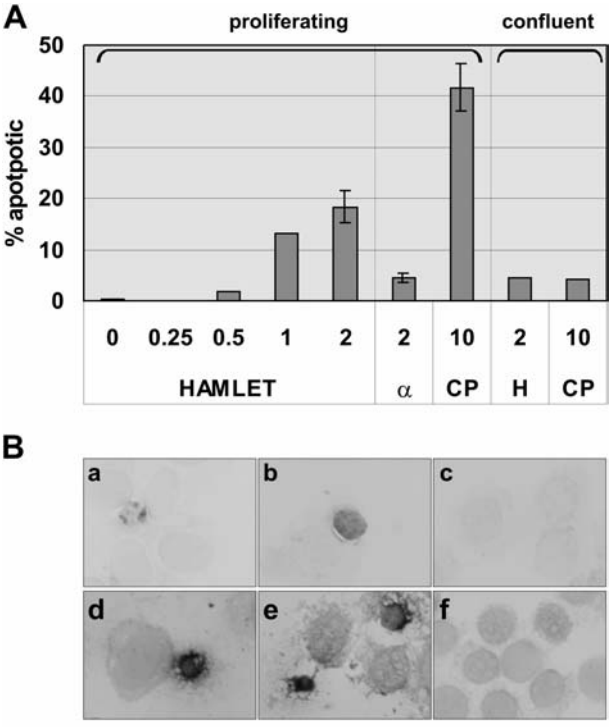

Figure 4. Apoptotic cell death of 31D mammary epithelial cells in response to HAMLET, α-LA or cisplatin (CP). (*A*) Exponentially growing cells were treated for 24 h with increasing concentrations of HAMLET (0.25–2 mg/ml) in the absence of fetal calf serum. Cells were collected, fixed and at least 300 nuclei were counted after DAPI staining and the percentage of condensed nuclei was determined. Similarly, condensed nuclei were counted when cells reached confluence before the addition of HAMLET (H). The 31D cells were similarly treated with inactive α-LA (α) and cell death determined after DAPI staining. As controls, proliferating and confluent cells were treated with 10 µg/ml CP. Standard deviations are shown for several data points. (*B*) Processing of procaspase-3 and the presence of active caspase-3 fragments was documented with the CM1 antibody (a) and DNA fragmentation was visualized after TUNEL staining in 31D cells treated with 2 mg/ml HAMLET (d). For comparison, 10 µg/ml CP resulted in CM1-positive cells (b) or TUNEL-positive nuclei (e). Panels c and f show CM1 and TUNEL staining of untreated cells.

Discussion

Programmed cell death is essential to remove secretory epithelial cells from the mammary gland during involution. The environmental factors that orchestrate this process are, however, poorly understood. This study investigated the local effect in the mammary gland of HAMLET, a folding variant of α-LA bound to oleic acid. HAMLET was shown to induce a collapse of alveolar structures (fig. 1) and proteolytic processing of procaspase-3 (fig. 2). In addition, HAMLET caused extensive fragmentation of chromosomal DNA (fig. 3). A weak apoptotic response to HAMLET was confirmed in vitro in a cell line derived from secretory epithelial cells. The effect was specific for HAMLET, as α-LA or oleic acid were inactive in these assays. The results suggest that milk constituents might serve as regulators of cellular involution in the mammary gland.

The molecular control of programmed cell death in the mammary gland has been studied in several systems. Caspase activation is fundamental to classical apoptosis and may be triggered by mitochondrial activation or by ligands binding to members of the Fas tumor necrosis factor (TNF) receptor family [26, 27]. This latter pathway is not essential in the mammary gland, however, as shown in Fas-deficient mice [28]. Still, the involvement of cytochrome c and caspase-3 was independently documented in SWISS/MORO mice [4], in Balb/c [6, 24] and in NMRI mice, suggesting that other mechanisms trigger the mitochondrial response. In Jurkat cells, HAMLET has been shown to associate with mitochondria and to trigger cytochrome c release and caspase-3 activation [19, 29]. The functional importance of the caspase-related apoptotic response to HAMLET has been questioned, however, as specific inhibitors like ZVAD did not inhibit cell death in vitro and overexpression of Bcl-2 did not rescue tumor cells from HAMLET-induced apoptosis, and thus the exact role of caspases for HAMLET-induced cell death in tumor cells needs further study. In the present study, in vivo activation of caspase-3 was observed, and only in tissues exposed to HAMLET. This is consistent with the tumor cell data, but does not prove that the caspase response is the cause of the morphological changes observed.

HAMLET induces apoptosis-like cell death by activation of several different pathways. One striking feature is the accumulation of HAMLET in the nuclei of tumor cells where it binds histones, disrupts chromatin structures and mediates nuclear DNA fragmentation [18, 29]. Consistent with this mode of action, the present study showed that HAMLET perturbed the chromatin structure in secretory epithelial cells in the mammary gland and in proliferating cells in culture. Apoptotic cell death during normal involution was previously shown to result in de novo expression of several genes, e. g. c-fos, c-jun and cyclin D that are typically expressed during G1 or during a G1-like state of the cell cycle. We therefore postulated previously that a partial dedifferentiation of secretory epithelial cells occurs before cells die by apoptosis [30]. Not clear presently is whether exposure to HAMLET initiates a similar dedifferentiation process before initiation of the apoptotic process occurs.

If HAMLET can accumulate in milk, and whether the conditions in the mature mammary gland may permit the formation of HAMLET from α-LA and oleic acid is not clear. Normal milk contains α-LA in native conformation and the physiological conditions required to remove the calcium and unfold the protein and form the active complex are presently not known. Low pH promotes these molecular changes. The conditions in the mammary

gland may change during involution, as milk synthesis ceases only gradually and milk accumulates in alveolar structures and may be exposed to low pH. Both conditions could favor the formation of α-LA folding intermediates and hydrolysis of triglycerides releasing the fatty acids. Due to the morphological situation in the mammary gland, secretory epithelial cells would be directly exposed to the milk molecules, thus providing a scenario where apoptosis of mammary epithelial cells is a self-regulating process which is induced or supported by milk components which accumulate in the gland after weaning. Until now, the mechanism by which HAMLET is internalized is not clear. Sealing of the central duct of a single gland has been shown to be sufficient to induce an apoptotic program exclusively in sealed glands while all other glands continue to be in a fully lactating state [7, 31], implying that the cell death program can locally dominate survival signals in the mammary gland.

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