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APOPTOSIS AND AUTOPHAGY INDUCED BY TGF-B1 IN BOVINE MAMMARY EPITHELIAL BME-UV1 CELLS

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Mammary gland growth and involution is based on a dynamic equilibrium between proliferation and apoptosis of mammary gland epithelial cells (MEC). TGF-ß1 is an important antiproliferative and apoptogenic factor for mammary gland epithelial cells, acting in auto/paracrine matter and thus considered an important local regulator of mammary tissue involution. So far the studies on mammary gland involution concerned only apoptosis as a type I of MEC programmed cell death (PCD). Autophagy is known to be type II of PCD and this paper is the first, supporting evidence for the TGF-ß1-induced autophagy in bovine mammary epithelial cell line BME-UV1, as a distinct to apoptosis type of PCD. Laser scanning cytometry and confocal microscopy were used for analysis of MAP1 LC3 and Beclin1 expression - two proteins considered being the most reliable biochemical markers of autophagy. The significant increase of MAP1 LC3 and Beclin1 expression in cells treated with TGF-ß1 (2 ng/ml) was observed. Ultrastructural observation in electron microscopy revealed that autophagy is not only alternative, but also complementary to apoptosis type of cell death in TGF-ß1-treated bovine MEC. It was manifested by typical morphological features of apoptosis (cell shrinkage, margination and condensation of chromatin) and autophagy (autophagosomes, autophagic vacuoles) in the same cell.

K e y w o r d s: *apoptosis, autophagy, Beclin1, BME-UV1 mammary epithelial cells, MAP1 LC3, PARP, TGF-ß1*

INTRODUCTION

Programmed cell death (PCD) is an essential physiological process operating at all stages of mammary gland remodelling. During mammary gland involution the extracellular matrix and the alveolar basement membrane are degraded. The alveoli loose their structural integrity and massive death of mammary epithelial cells (MEC) is observed. Apoptotic cell death is responsible for cell loss during mammary gland involution after natural weaning or litter removal in rodents (1, 2), after weaning in sow (3) and during drying off in goat (4) and cow (5).

So far the studies on mammary gland involution concerned only apoptosis as a way of MEC death in secretory tissue. However accumulating evidence suggest that apoptosis is not the only type of programmed cell death (PCD). Cells use different pathways to activate self-destruction process and nowadays tree types of PCD have been distinguished. Type I PCD - apoptosis depends on activation of caspases and is characterized by typical morphological features, such as: cell shrinkage, condensation of chromatin, pycnosis and fragmentation of the nucleus, formation of apoptotic bodies. Type II PCD - autophagy is characterized by accumulation of autophagic vacuoles. Type III PCD, or cytoplasmic death is less frequently observed and poorly understood at the molecular level. There are first evidence that the Type II PCD is also observed in mammary epithelial cells. Mills et al. (6) used an *in vitro* morphogenesis model in which MCF-10A human mammary epithelial cells form hollow acini-like structures and observed both: caspase-mediated apoptosis and autophagy associated with cells that are lost during lumen formation.

Autophagy is thought to play a major role in intracellular degradation. During the autophagic process, a single-membrane structure (isolation membrane) surrounds portion of the cytoplasm and organelles. Fusion of the tips of the isolation membrane to each other forms a double-membrane spherical autophagosome, which in the next step fuses with lysosomes and the sequestrated contents, and the inner membranes are degraded by lysosomal hydrolases. Some conditions, including starvation and hormonal stimulation, can enhance autophagy dramatically. If mammalian cells are cultured for 2 hours without amino acids and serum, the ratio of autophagosomes area to total cytoplasmic area increases by about 10 times, which was observed in cultured mouse embryonic stem cells (7) and HeLa cells (8).

Main genes involved in autophagy regulation in mammalian cells are APG (autophagy) (9) and AUT (autophagocytosis) (10). MAP1 LC3 (microtubule-associated protein 1 light chain 3) is a mammalian homolog of yeast Aut7/Apg8, which exists on the autophagosome membrane and isolation membranes (8, 11). MAP1 LC3 is currently the only reliable marker of autophagosomes presence (12). Another important protein involved in the type II PCD is Beclin1, which is required for vacuolar transport and autophagy (13), and acts as a suppressor of tumorgenesis. Beclin1 forms a complex with the

class III phosphatidylinositol 3-kinase (class III PI3K), which is also involved in autophagy (14). This complex was found in the *trans*-Golgi network (TGN), suggesting the possibility of autophagy control by providing PtdIns 3-phosphate (PtdIns3P) from the TGN to the isolation membranes. Beside class III PI3K, Beclin1 has been shown to interact with Bcl-2 (15) - a protein involved in the control of apoptosis. This interaction is required to protect murine neurons infected by the Sindbis virus from cell death. It is still not known whether the Beclin1/Bcl-2 interaction is instrumental in autophagy, however the fact that autophagy is stimulated when Bcl-2 is down regulated has to be emphasized (16). Autophagic cell death has been described in anti-estrogen-treated cultured human mammary carcinoma MCF-7 crells (17). This cell death is also inhibited by estradiol, indicating that tamoxifen-induced cell death is not just a passive necrotic toxicity. Tamoxifen stimulates the expression of Beclin1, which supports its stimulatory effect on autophagy (18).

The distinction between type I and type II PCD is not always clear. Autophagy can be instrumental in type I cell death - apoptosis, and there are several cases of type II cell death where some traits of apoptosis have been observed. Apoptosis and autophagy are probably evolutionary-related processes (19). This has recently received strong support by the demonstration, in mammalian cells, that the Ca^{2+} / calmodulin-regulated death kinases DAPk and DRP-1 control both: autophagic cell death and the membrane blebbing - characteristic of apoptosis (20). The formation of autophagosomes has been shown to be associated with TNFα-dependent apoptosis in human T-lymphoblastic leukaemic cells (21). A molecular link between apoptosis and autophagy was suggested when the cloning of mammalian protein ASP (apoptosis-specific protein) revealed its similarity to the yeast Apg5 (22). Although ASP transcripts are present in viable and apoptotic cells, the polypeptide is only detected in the late stage of apoptosis. However the human Apg5 protein has been shown to be expressed in conditions where apoptosis is not induced (23). Autophagic cell death has also been described when caspases are inhibited or in conditions in which expression of Bcl-2 family members has been manipulated. Expression of Bax, a cell death agonist of Bcl-2 family in Jurkat T-cells is sufficient to trigger apoptosis (24). However, blocking caspases did not prevent the Bax-induced cell death, which turned to an autophagic cell death. The presence of Bax at the surface of mitochondria could suggest a role of this organelle in the autophagic cell death program. Anti-sense downregulation of Bcl-2 protein in human leukemic HL-60 cells induces an autophagic cell death program, which is not rescued by caspase inhibitors and which does not depend upon cell death mitochondrial signaling (15). Apoptosis and autophagic cell death can also share common inhibitory or activating signaling pathways in mammalian cells and has been recently demonstrated for Akt/PKB (25) and mTOR (26).

Because of growing evidence of the close relationship between apoptosis and autophagy we decided to examine whether the type II programmed cell death

occurs also during mammary gland remodelling. Many studies have shown that mammary gland epithelial cells apoptosis seems to be induced by the following intramammary signals such as: physical distension of epithelium (27); feedback regulation by factors present in milk e.g. FIL - feedback inhibitor of lactation (27); increase of local growth inhibitors and inductors of apoptosis secretion by mammary epithelial cells e.g.IGFBP-3 (IGF binding protein -3), IGFBP-5, ligand Fas, MDGI (mammary derived growth inhibitor) and TGF-ß1 (transforming growth factor - ß1) (28-31). Our studies were mainly concentrated on the last cytokine - TGF-ß1.

TGF-ß1 is an antiproliferative factor for MEC acting in an auto/paracrine manner and thus considered an important local regulator of mammary tissue involution. It has been shown that TGF-ß1 inhibits proliferation, differentiation and milk protein synthesis in MEC and increases extracellular matrix formation (32). TGF-ß1 is also an apoptogenic agent for MEC, which has been shown in mouse and bovine mammary gland (31, 33-36). The results of our study (35, 37) have shown that TGF-ß1 triggers apoptosis in mouse and bovine MEC through a mitochondrial pathway involving: activation and translocation of Bax to mitochondrial membranes, release of cytochrome c, activation of caspase-3 and degradation of its substrate - poly(ADP-ribose)polymerase (PARP) in the nucleus. We have also confirmed that TGF-ß1-induced apoptogenic signal is transduced into the nucleus by Smad proteins, which act as transcription factors on promoters of target genes. Our study have shown that one of those target genes might be IGF binding proteins, which further mediate the signal causing IGF-I sequestration and inhibition of PKB/Akt-dependant survival pathway (36).

The present paper is the first, supporting the evidence for the TGF-ß1-induced autophagy in bovine mammary epithelial cell line BME-UV1, shown by an increase of MSP1 LC3 and Beclin1 expression and characteristic ultrastructural features. This may prove that TGF-ß1 is not only involved in MEC apoptosis, but also induces type II of cell death - autophagy and both types of PCD occur simultaneously in bovine mammary epithelial cells.

MATERIALS AND METHODS

Media and reagents

DME/F-12, RPMI-1640, NCTC 135 powdered medium, α-lactose, lactalbumin hydrolysate, glutathione, bovine insulin, bovine holo-transferin, hydrocortisone, L-ascorbic acid, transforming growth factor-beta1 (TGF-ß1) and all the other reagents were from Sigma Chemicals Corp. (St. Louis, MO). Polyclonal and monoclonal antibodies against: MAP1 LC3, Beclin1, PARP - poly(ADP-ribose)polymerase, were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Alexa Fluor 488 secondary antibodies were purchased from Molecular Probes (Eugene, OR). Plastic cell culture Petri dishes were purchased from Corning Glass (Corning, NY).Sterile conical flasks, disposable pipettes were supplied by Nunc Inc.(Naperville, IL).

Cell culture

The bovine mammary epithelial BME-UV1 cell line was established by Professor B. Zavizion (Department of Animal and Food Sciences, University of Vermont, USA) and was kindly provided by Professor Antonella Baldi (Animal Nutrition Institute, Faculty of Veterinary Medicine, University of Milan, Italy).

Cells were cultured in routine culture medium (mixture of DME/F-12, RPMI-1640 and NCTC 135 in proportions of 5:3:2 by vol.) enriched with α-lactose (0.1%), glutathione (1.2 mM), bovine insulin (1.0 µg/ml), bovine holo-transferrin (5.0 µg/ml), hydrocortisone (1.0 µg/ml), L-ascorbic acid 10 µg/ml), 10% (v/v) heat-inactivated FBS, penicillin-streptomycin (50 IU/ml), fungizone (2.5 µg/ml), gentamycin (50 µg/ml) in atmosphere of 5% CO_2 / 95% humidified air at 37°C and routinely subcultured every 2 days.

Experimental procedure and immunofluorescence staining for laser scanning cytometry

Cells were cultured on Lab-Tek two-chamber slides until confluency in a routine culture medium (see above). The medium was then removed and replaced with 0.5% FBS medium for 24 h. Next experimental factor: TGF-ß1 (2 ng/ml) was administered to FBS-deficient cells for 3, 6, 12 and 24 h. The concentration of TGF-ß1 used in the experiments was established after screening tests and on the base of previous experiments (40, 42, 43). Cells not exposed to experimental factors were used as a control to each experiment. For each experiment at least three replicates were performed.

The cells were fixed in 0.25 % formaldehyde for 15 min, washed twice with PBS, suspended in ice-cold 70 % methanol and stored at 4°C for 30 min. Finally methanol was aspirated and samples were stored in -80°C until staining.

The cells were washed twice with PBS-1% bovine serum albumine (BSA) and incubated for 1 h with primary antibodies diluted 1:250 with PBS-1% BSA. After primary incubation the cells were washed twice with PBS-1% BSA and incubated for 1 h with 1:500 (Alexa Fluor 488) secondary antibodies. The cells were then washed twice in PBS-1% BSA and finally incubated in 5 µg/ml solution of 7-aminoactinomycin D (7-AAD) for 30 min to counterstain the DNA. Finally the coverslips were mounted on microscope slides using ICN mounting medium (ICN Biomedicals inc,. Aurora, OH).

Laser scanning cytometry

Probes were analyzed by LSC (CompuCyte Corp., Boston, MA). At least $5x10^3$ cells per slide were analyzed. The fluorescence excitation was provided by a 488 nm argon laser beam. A combination of dichroic mirrors and filters transmitting light at a $520 \pm 20nm$ wavelength for green fluorescence of Alexa Fluor 488 antibodies, and >650 nm for far red fluorescence of 7-AAD was used. Green fluorescence was measured separately over the nucleus (NF) and the cytoplasm (CF). NF was measured within the area outlined by the 'integration contour', located 2 pixels outside the 'threshold contour' triggered by the far red fluorescence of 7-AAD. CF was measured within the rim of cytoplasm 10 pixels wide, located outside the 'integration contour'. The background green fluorescence was automatically measured within a 2 pixels range outside the 'peripheral contour' and subtracted from both, nuclear and cytoplasmic green fluorescence, to obtain the final values of NF and CF, respectively. Another parameter measured was the high maximal pixel (HMP) corresponding to the highest value of measured fluorescence in the cell, regardless of the cellular compartment.

Confocal microscopy

Cells were stained according to immunofluorescence staining procedure described in LSC section. Next they were visualized by confocal laser scanning microscope FV-500 system (Olympus Optical Co, Hamburg, Germany). The combination of excitation/emission were: Argon 488 nm laser with 505-525 nm filter for Alexa Fluor 488 and HeNe 543 nm laser with 610 nm filter for 7AAD nucleus staining. Stack of cross-sections from representative control and TGF-ß1-treated cells were gathered separately for each fluorescence channel. 3D images and series of cross-sections were reconstructed using Fluoview program (Olympus Opitcal Co).

Electron microscopy

For ultrastructural studies the BME-UV1 cells were fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in 0,1 M PBS for 1 hour at 4°C. The cells were washed in PBS for 30 min, treated with 1% OsO_4 for 1 hour, dehydrated in ethanol gradient and embedded in Epon. For electron microscopy, ultrathin sections were processed according to the post-embedding procedure. Briefly, the sections were mounted on the formvar-coated nickel grids, incubated in 10% hydrogen peroxide for 10 min, rinsed in PBS for 15 min and blocked for 15 min in 5% bovine serum albumin (BSA) in PBS (PBS-5% BSA). The material was air-dried, stained for 10 min with 4.7 % uranyl acetate and for 2 min with lead citrate. The sections were examined and photographed in JOEL 1200EX electron microscope.

Statistical evaluation

The results were statistically evaluated by ANOVA and Tukey's multiple range tests using Prism version 2.0 software (GraphPad Software, San Diego, CA). P≤ 0.05 was regarded as significant and P≤0.01 as highly significant.

<div align="center">RESULTS</div>

Apoptotic effect of TGF-ß1 on BME-UV1 bovine mammary epithelial cells

TGF-ß1 (2 ng/ml) was administered to cells grown in a FBS-deficient medium for 6, 12 and 24 h to show the apoptotic effect of the cytokine. Cell cycle and percent of apoptotic cells were measured using LSC. Cells not exposed to experimental factors were used as a control to the experiment. Apoptotic cell number was evaluated by the measurement of cell number in sub-G1 region on DNA histogram (cells with lowered DNA content). TGF-ß1-treatment resulted with highly significant increase of apoptotic cell number, reaching the peak after 12 h (*Fig.1a*). The apoptotic effect of TGF-ß1 was confirmed by measurement of PARP expression (89 kDa degradation product) - a typical sign of caspases-3 proteolytic activity (*Fig.1b*). Administration of TGF-ß1 evoked highly significant increase in PARP-related fluorescence, which preceded the increase of apoptotic cell number, measured in sub-G1 region (*Fig.1a*). The changes in cell cycle are presented as G1/(S+G2/M) ratio (*Fig.1c*). A highly significant decrease in the ratio was observed after 12 h incubation with TGF-ß1 in comparison to control cells, which were not treated
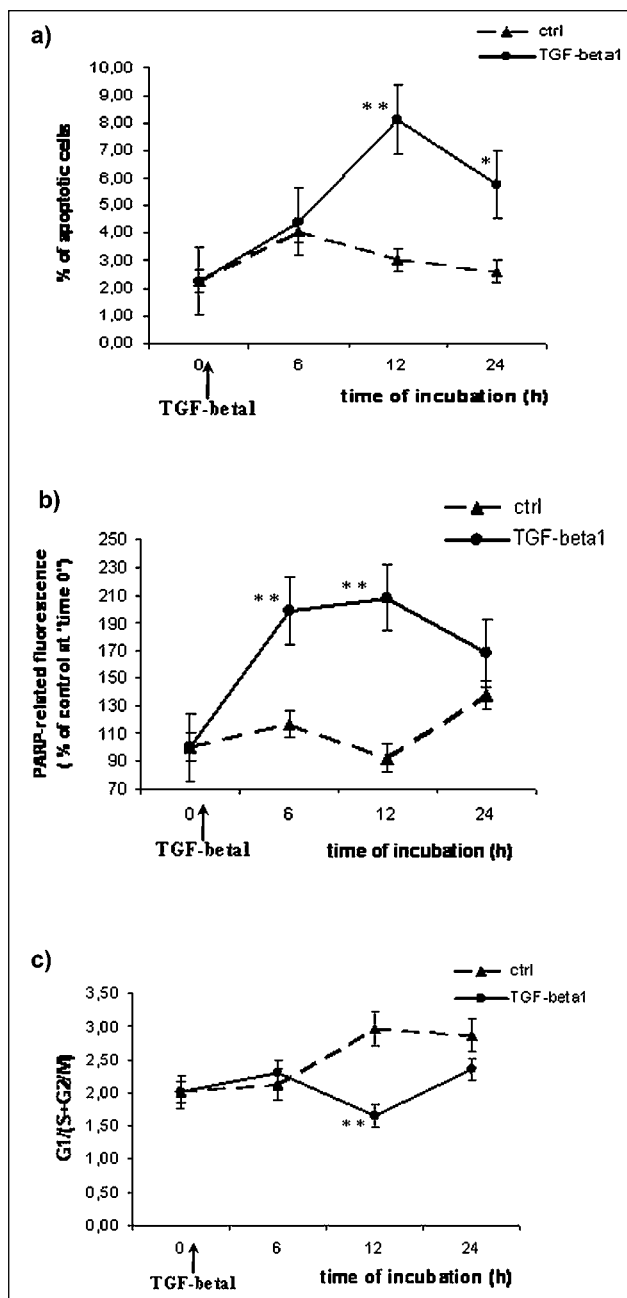
Fig. 1. Antiproliferative and apoptotic effect of TGF-ß1 (2 ng/ml) in bovine mammary epithelial cell line BME-UV1, measured by LSC. Cell culture was treated with the cytokine for 6, 12 and 24h. Cells not exposed to TGF-ß1 represent the control. The TGF-ß1 effect is shown as: a) percentage of cells in subG1 region; b) changes in the level of 89 kDa PARP - poly(ADP-ribose)polymerase - degradation product, c) changes in cell cycle presented as G1/(S+G2/M) ratio. Results are presented as means ± SEM from three separate experiments.
* - significant difference (p≤0.05) in comparison with control culture; ** - highly significant differences (p≤0.01) in comparison with control culture.

with the cytokine, but were grown in FBS-deficient medium. This effect coincided with the peak of apoptosis in TGF-ß1-treated culture (*Fig.1a*) and was probably due to the shift of cells from G1 to sub-G1 region.
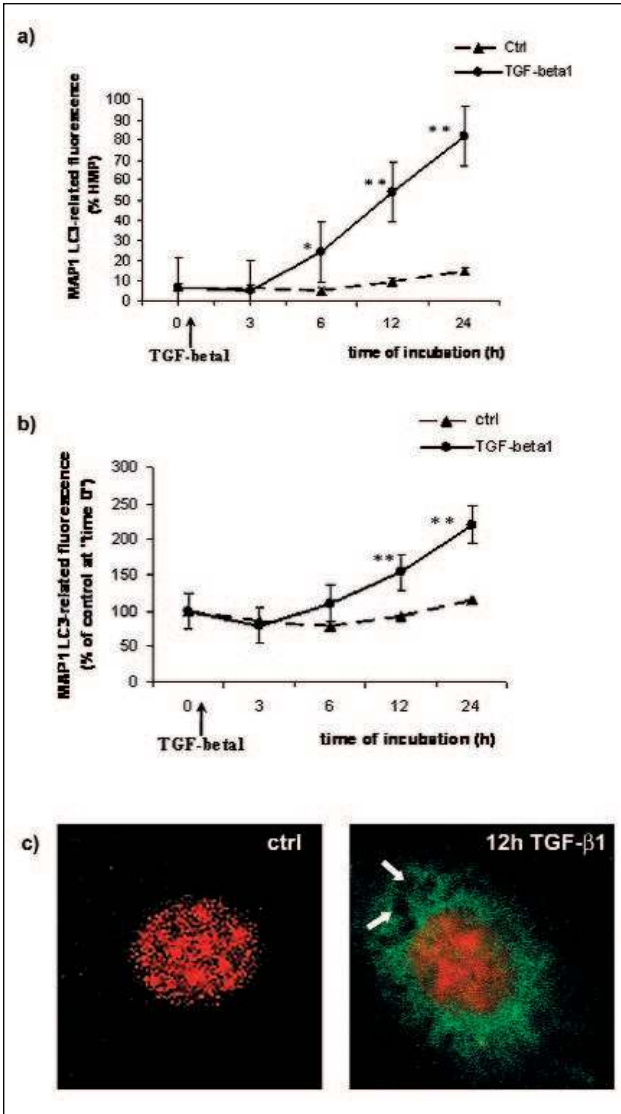
Fig. 2. TGF-ß1-induced MAP1 LC3 expression in BME-UV1 bovine mammary epithelial cells, measured by LSC and presented as: a) percentage of cells with HMP (high maximal pixel corresponding to the highest values of fluorescence in cells); b) MAP1 LC3-related fluorescence shown as percent of control at "time 0", where "time 0" represents control cell culture before cytokine administration; c) visualization of MAP1 LC3 expression on confocal image in representative cross-sections of cell exposed to TGF-ß1 (2 ng/ml) for 12 h and control cell, not treated with the cytokine. Autophagic vacuoles are pointed by arrows.
In the experiment TGF-ß1 (2 ng/ml) was administrated to FBS-deficient medium (with 0.5% FBS) and cells were treated with the cytokine for 3, 6, 12, and 24h. Cells not exposed to TGF-ß1 represent the control. Results are presented as means ± SEM from three separate experiments.
* - significant difference (p≤0.05) in comparison with control culture; ** - highly significant differences (p≤0.01) in comparison with control culture.

TGF-ß1 influence on MAP1 LC3 and Beclin1 expression

To examine whether the type II of programmed cell death - autophagy may occur during mammary gland remodelling, the expression of two characteristic for autophagy proteins was measured. BME-UV1 bovine MEC were maintained in FBS - deficient medium. Cells were treated with TGF-ß1 (2 ng/ml) for 3, 6, 12, 24 hours. Not treated, control cells were sustained in the FBS-deficient medium in the same experimental period. TGF-ß1 evoked a

highly significant increase in MAP1 LC3 expression measured as percent of cells with HMP (high maximal pixel) and MAP1 LC3-realted fluoresce (*Fig.2 a, b*). The increase in expression was visualized on confocal image in representative cross-sections of cell exposed to TGF-ß1 (*Fig.2c*). For comparison visualization of a cell not treated with the experimental cytokine was also performed. Representative control cell did not show any green fluorescence corresponding to MAP1 LC3 expression. Only nucleus labeled by 7-AAD, and therefore emitting red fluorescence, was detected. In cell treated with TGF-ß1 high MAP1 LC3 expression was revealed as a strong green fluorescence emitted as a result of excitation of green fluorescence dye. Empty areas within MAP1 LC3-related green fluorescence were observed, indicating the presence of autophagic vacuoles (*Fig. 2c*).

Beclin1 expression was also elevated in cells exposed to TGF-ß1. The highest expression was observed after 12 h of incubation with TGF-ß1 but after this time the decrease of Beclin1 level to control value was observed (*Fig. 3a, b*).
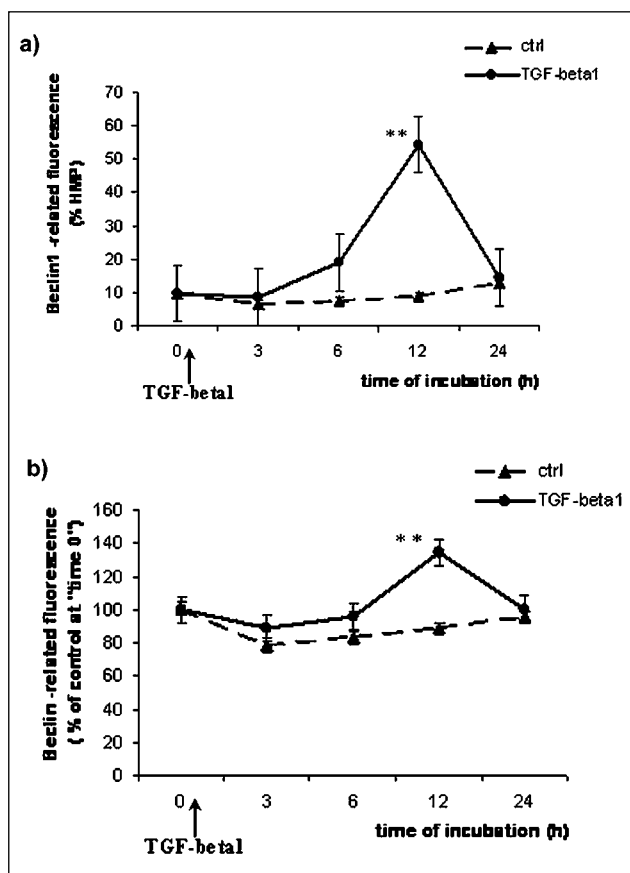

Fig. 3. TGF-ß1-induced Beclin1 expression in BME-UV1 bovine mammary epithelial cells measured by LSC, and presented as: a) percentage of cell with HMP (high maximal pixel corresponding to the highest values of fluorescence in cells); b) Beclin1-related fluorescence shown as percent of control at "time 0", where "time 0" represent control cell culture before cytokine administration. In the experiment TGF-ß1 (2 ng/ml) was administrated to FBS-deficient medium (with 0.5% FBS) and cells were treated with the cytokine for 3, 6, 12, and 24 h. Cells not exposed to TGF-ß1 represent the control. Results are presented as means ± SEM from three separate experiments. ** - highly significant differences (p≤0.01) in comparison with control culture.

Morphological features of TGF-ß1-induced apoptosis and autophagy in bovine BME-UV1 MEC

The healthy BME-UV1 cells showed normal ultrastructural morphology. The oval, uniform nuclei which were delimited by a double membrane - nuclear envelope with nuclear pores were present. In nucleus dispersed or small irregular clumps of chromatin and one or two electron dense nucleoli were present. The
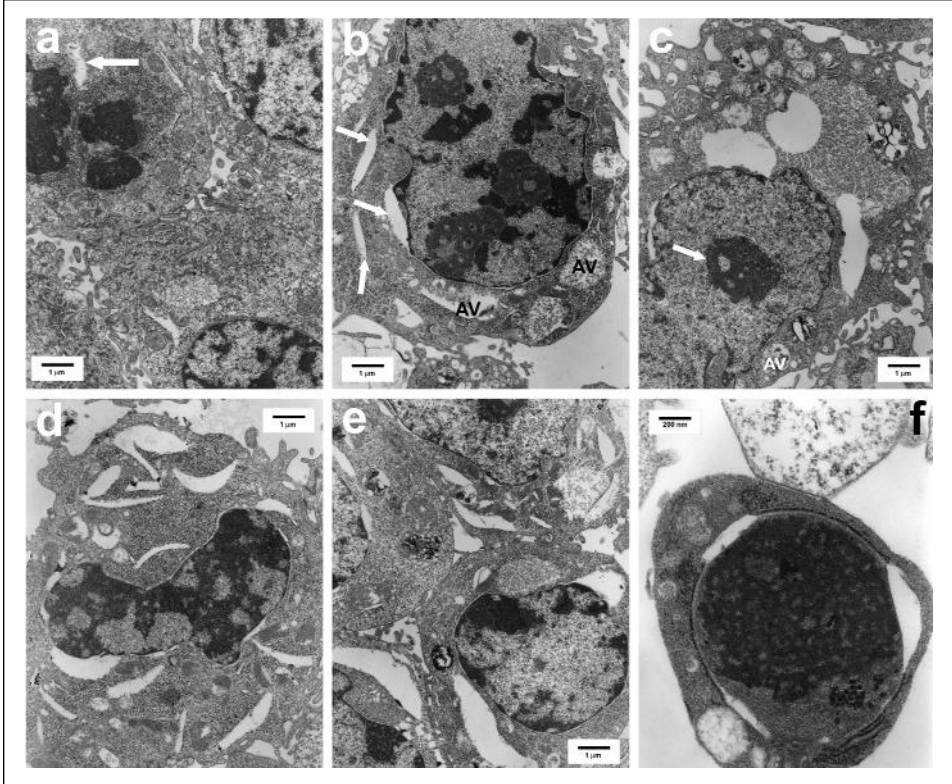

Fig. 4. Examples of morphological phenotypes variety of cell death in BME-UV1 bovine mammary epithelial cells treated with TGF-ß1 for 12 h: a) fragments of three ultrastructurally unchanged control cells, one of them exhibits mitosis (arrow); b) typical autophagic appearance of dying cell after 12 h of TGF-ß1-treatment: in nucleus, dispersed chromatin and multiplication of nucleoli in association with condensed chromatin; in condensed cytoplasm formation of electron-lucent hollow acini-like structures (arrows) and autophagic vacuoles (AV); c) figure demonstrating autophagic cell with many autophagic vacuoles (AV), with range of morphologies and large hollow acini-like structures; microsegregation of nucleoli (arrow), swollen mitochondria and lack of polyribosomes; d) dying cell with a morphology characteristic for apoptosis (condensation of chromatin, pycnotic nucleus, plasma membrane blebbing) and autophagy hollow acini-like structures, affected mitochondria); e) picture demonstrating three cells with whole spectrum of morphological features of apoptosis and autophagy together; f) an oval, very small, apoptotic body with chromatin condensation, which is segregated into crescent or cap; dilatation in the space between the inner and outer part of the nuclear envelope can be observed.

cytoplasm was rich in organelle: RER, ribosomes, polyribosomes, very well developed Golgi complex, mitochondria, small number of lysosomes, dense bodies, multivesicular bodies and occasionally present autophagolysosomes. The cytoskeleton appeared ultrastructurally normal. The plasma membrane with characteristic broadened processes was present. (*Fig. 4a*)

In some cell populations, 12 h after TGF-ß1-treatment, the most of the cells became vacuolated, exhibiting type II of PCD. The prime subcellular region for early vacuoles or hollow acini-like structures of various dimension tended to be perinuclear. A striking but important ultrastructural feature in BME-UV1 cells was their apparently membranelles appearance and lack of obvious electron-dense content or sometimes content of a few granular electron-light materials. Decreased polyribosomes and increased ribosomes density and typical, autophagic vacuoles with a range of morphologies in condensed cytoplasm were present. The degeneration of Golgi complex and ultrastructural changes in mitochondria were obseved. In these cells intact cytoskeleton was present. In nucleus with irregular contour partial chromatin condensation, or marginalization to the nuclear envelope were observed. Vacuolar dilatations were observed in the space between the inner and the outer part of the nuclear envelope. In some cells the level of chromatin increased and showed a tendency to aggregate around the nucleoli (*Fig. 4b, c*). Between such cells, apoptotic cells exhibiting the most characteristic features of the type I PCD were observed. However a whole spectrum of morphological features representing apoptosis and autophagy was seen in degenerating cells (*Fig 1 d, e, f*). The late apoptotic cells and apoptotic bodies could be identified by condensed texture and crescent shape of their chromatin TGF-ß1-treated cultures (*Fig. 4f*).

DISCUSSION

In spite of the great interest in the function of bovine mammary gland, the molecular mechanisms responsible for mammary gland remodelling are still unclear. It is commonly accepted that physiological base of mammary gland growth and involution is a dynamic equilibrium between proliferation and apoptosis of MEC. TGF-ß1 is a major auto/paracrine antiproliferative and apoptogenic agent in bovine MEC (35, 36). Expression of this cytokine in bovine MEC is under the control of somatotropic axis hormones and increases during dry period (38).

Present paper is the first showing, that TGF-ß1 induces not only apoptosis (PCD I), but also autophagy (PCD II) in bovine MEC. Up to now the occurrence of autophagy as an alternative to apoptosis death pathway in bovine mammary gland has not been documented. TGF-ß1-induced autophagy is manifested with the increase of MAP1 LC3 (*Fig. 2*) and Beclin1 (*Fig. 3*) expression. Both of these proteins are currently the most reliable biochemical markers of autophagy (12).

The increase of Beclin1 level was transient, reaching the peak at 12 h after TGF-ß1 administration (*Fig. 3*). Beclin1 gene, the mouse homologue of yeast ATG-6, encodes a Bcl-2 interacting candidate tumour suppressor and antiviral protein. Ultrastructural analysis of cells with reduced Beclin1 protein levels showed a parallel inhibition of autophagic vacuole formation associated with reduced cell death (39). These results indicate that Beclin1 is indispensable to trigger autophagic pathway of cell death. It has been shown, that Beclin1 forms a complex with the class III phosphatidylinositol 3-kinase (PI-3K), which was found in the *trans*-Golgi network (TGN), suggesting the possibility that the complex Beclin1-PI-3K controls autophagy by providing PI-3P from the TGN to the isolation membranes (40).

Comparison of the kinetics of TGF-ß1-induced apoptosis and autophagy, indicates that the peak of apoptosis, measured by sub-G1 region (*Fig. 1a*) and 89 kDa PARP degradation product (*Fig. 1b*) occurs at 12 h of TGF-ß1 treatment, whereas autophagy evaluated by MAP1 LC3 expression increases progressively throughout 24 h of the experiment (*Fig. 2*). Moreover, the peak of apoptosis at 12 h corresponds to the decrease in G1/(S+G2/M) ratio, suggesting that apoptotic cells originate from G1 phase of cell cycle in TGF-ß1treated cultures (*Fig. 1c*). These results are concordant with our earlier study, showing that TGF-ß1-induced apoptosis in mouse leukaemic L1210 cells concern first of all cells being in G1 phase of cell cycle (41). Presented results indicate, that apoptosis is more dynamic process than autophagy in TGF-ß1 treated bovine MEC. Similar results were obtained in camptothecin (CPT)-treated human breast cancer MCF-7 cells, where the peak of apoptosis was observed within 1h after CPT administration, whereas autophagy increased progressively during 24 h of the experiment (42). Ultrastructural observations revealed that autophagy is not only alternative, but also complementary to apoptosis type of cell death in TGF-ß1-treated bovine MEC. It was manifested with typical morphological features of apoptosis (cell shrinkage, margination and condensation of chromatin) and autophagy (autophagosomes, autophagic vacuoles) in the same cell (*Fig. 4b, d, e*). It is possible that the faster program of apoptosis eliminates the most sensitive cell subpopulations, whereas the remaining, more resistant cells develop a slower program of autophagy as complementary to apoptosis type of cell death. Complementarity of apoptosis and autophagy suggests mutual interconnections between both PCD pathways, however the molecular switch still remains unidentified. Several molecular links between apoptosis and autophagy have been suggested in various cellular models, including: ASP (apoptosis-specific protein), Beclin1, Ca^{2+}/calmodulin-regulated death kinases DAPk and DRP-1, PTEN, steroid-inducible gene *E93*, signaling molecules Akt/PKB and mTOR, and Bcl-2 family proteins (43). Our recent results revealed that BID, being the major protein controling mitochondrial membrane permeability in the course of apoptosis may serve as molecular switch between apoptosis and autophagy (42). It was proved by "knock down" of BID, which

resulted in strong suppression of CPT-induced apoptosis and a shift towards autophagy in breast cancer MCF-7 cells, manifested with significant increase of Beclin1 and MAP1 LC3 expression.

In conclusion: 1) TGF-ß1 induces both apoptosis and autophagy in bovine BME-UV1 cells; 2) TGF-ß1-induced autophagy manifests with increased levels of Berclin1 and MAP1 LC3 proteins and ultrastructural features, like autophagosomes and autophagic vacuoles; 3) Autophagy is not only alternative but also complementary to apoptosis type of PCD in bovine MEC exposed to TGF-ß1.

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