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Functional expression of bcl-2 protein family and AIF in bovine mammary tissue in early lactation

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Cell proliferation and apoptosis were measured in bovine mammary tissue around the time of peak milk production in three heifers, and compared with changes in the expression of bcl-2-related intracellular signals and apoptosis-inducing factor (AIF). Cell proliferation and apoptosis were relatively constant during the study period with no significant change in the incidence of either event. However, the ratio of apoptotic to proliferating cells tended to change from 0·99 on day 45 of lactation to 1·82 on day 63 ($P=0·064$), suggesting that in the course of the study, a dynamic balance may have been succeeded by net cell loss. Average milk production recorded 6 d after biopsy was correlated with the estimated number of cells ($r=0·898$; $P<0·01$) but not with the apoptosis to proliferation ratio ($r=-0·224$, $P>0·05$). Turnover of the cell population was associated with relatively constant expression of anti-apoptotic bcl-2. Competitive PCR also indicated expression of bax, in contrast to observations in lactating rodent mammary tissue. Bax expression was relatively low compared with that of bcl-2, but immunohistochemical staining for bax protein, which was not detectable on day 45, was observed on day 53 and, more intensely, on day 60 when the protein appeared to be membrane-associated. A partial coding sequence for bovine AIF was identified and AIF expression was evaluated by *in situ* hybridization. The results indicated that AIF was expressed in luminal alveolar cells and that, in concert with a change in bax to bcl-2 ratio, they might contribute to signalling of a change in the dynamic balance of the cell population as lactation progresses.

Keywords: Cow, lactation, bcl-2, AIF, *in situ* hybridization.

Apoptosis is important in mammary development (Strange et al. 2001), and in the tissue's involution after lactation (Capuco & Akers, 1999; Wilde et al. 1999). Control of lactation may also involve apoptosis: in dairy animals, as milk yield declines progressively after peak lactation, tissue adaptation includes epithelial apoptosis (Wilde et al. 1997; Li et al. 1999). One of the most important regulators of apoptosis is the family of bcl-2 proteins (Reed, 2000). The mechanisms through which these proteins, which include anti-apoptotic proteins bcl-2 and bcl-X_L and pro-apoptotic bax and bcl-X_S, regulate apoptosis are not completely defined (Schorr et al. 1999), but involve changes in the permeability of the mitochondrial membrane. Bcl-2 resides in the outer membrane, which is the site at which bax and bcl-2 can homo- or hetero-dimerize via the BH3 domain (Mahajan et al. 1998). Bcl-2 members contain a pore-forming domain (Reed, 1997) which could control the formation of permeability transition pores in the mitochondrial membrane. Apoptosis-Inducing Factor (AIF), a recently identified flavoprotein containing an oxidoreductase domain, acts as a mediator of apoptosis (Hisatomi et al. 2001). AIF is normally confined to mitochondria, but is stimulated by apoptotic factors to translocate to the cytosol and nucleus (Crompton, 1999; Susin et al. 1999).

The roles of the bcl-2 proteins and their interaction with AIF during mammary involution are emerging from studies in rodents (Gilmore et al. 2000). In contrast, little is known about the role of these proteins in lactating mammary tissue, particularly in large animals. The present study describes the incidence of mammary apoptosis in dairy cows at a stage of lactation when the cell population is in dynamic balance, i.e., after post-calving cell proliferation and before net cell loss after peak lactation, and identifies a number of apoptosis-related signals associated with these events. The study confirms apoptosis as an integral process

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in lactating bovine mammary tissue, and shows its association with expression of bcl-2 family members and, for the first time, bovine AIF.

Material and Methods

Animals and samples

Three first-calving Holstein Friesian cows were used. Clinical examination showed the heifers to be healthy and not affected by mastitis. Animals were housed in a small free-stall, and fed a total mixed ration (TMR) twice daily, at 8.00 and 16.00 in amounts sufficient to ensure that intake was *ad libitum*. The TMR contained, on a dry matter basis, 29·7% corn silage, 22·0% ground corn, 13·5% alfalfa hay, 6·8% cotton seeds, 6·8% grass hay, 6·1% solvent-extracted soyabean meal, 5·3% brewer's grains, 5·2% protein, mineral and vitamin supplement and 4·6% dried lucerne. Morning and evening milk yields were recorded every 3 d. Mammary gland tissue was sampled using a Bard biopsy instrument, mounted with a 12 gauge needle (Magnum Biopsy System, Bard, Convington GA 30209 USA) under subcutaneous anaesthesia from the right-rear quarter of the udder. Serial biopsies (20–30 mg of tissue) were collected from the three heifers 2 h after the morning milking 45, 53 and 60 d after calving. The biopsy site was treated with antiseptic liquid and antibiotic for 2 d, and after 48–72 h no blood or blood clots were seen in milk. Animals recovered milk yield in the subsequent 2 d and showed no clinical signs of disease. Immediately after biopsy, tissue samples were frozen in liquid nitrogen or fixed in 10% neutral formalin. The experiment was carried out in accordance with state and local laws and ethical regulations.

Histology, TUNEL method and immunohistochemistry

Histological sections (5 μm) were stained with haematoxylin-eosin, or processed for detection of cell death *in situ* by terminal dUTP nick end labeling (TUNEL) (Frag-EL Kit, Oncogene Research Products, Boston, MA, USA). Sections were incubated in 3% hydrogen peroxide in methanol for 10 min to inhibit endogenous peroxidases. Sections were treated with trypsin-$CaCl_2$ (0·5% w/v) for 15 min at 37 °C, and incubated overnight at 4 °C with rabbit polyclonal antiserum against bax protein (Chemicon Int., Temecula, CA, USA) or bcl-2 protein (Chemicon Int., Temecula, CA, USA). Slides were washed with Tris-buffered solution (TBS) and incubated with biotin-conjugated goat anti-rabbit IgG diluted 1 : 100 in PBS (Vector, Burlingame, CA, USA). After incubation in ABComplex (Vector), the stain was developed with 0·04% (w/v) diaminobenzidine tetrahydrochloride (Sigma, St. Louis MO, USA) in TBS containing 0·04% (v/v) hydrogen peroxide for 7 min at room temperature. In control sections, PBS containing 10% (v/v) normal goat serum and 1% (w/v) bovine serum albumin (BSA) was substituted for the first antibody.

Table 1. Upstream (for) and downstream (rev) primers for bovine bcl-2, bax, bcl-x cDNA amplification

Gene and Genebank accession number	Primer sequence	cDNA (bp)
bcl-2 U92434	for 5′-ATGTGTGTGGAGAGCGTCAA-3′ rev 5′-CAGACTGAGCAGTGCCTTCA-3′	201
bax U92569	for 5′-TTTGCTTCAGGGTTTCATCC-3′ rev 5′-TGGGTGTCCCAAAGTAGGAG-3′	431
β-actin AY141970	for 5′-CTAGGCACCAGGGCGTAAT-3′ rev 5′-CACACGGAGCTCGTTGTAGA-3′	176

PCNA monoclonal antibody (Chemicon Int., Temecula, CA, USA) was used as a marker of cell proliferation. The method was similar to that described for bax and bcl-2, except that antigen retrieval was omitted and PBS was used instead of TBS as incubation buffer.

Cell counting

Total cell number, PCNA-positive cells and TUNEL-positive cells were scored in ten microscopic fields (area of 0·24 × 0·24 mm) from each tissue sample under × 40 magnification. Indices of apoptotic and proliferating cells were calculated as the mean ratio of the number of TUNEL- or PCNA-positive cells to total cell number in each set of tissue sections.

RNA extraction and primer design

Total RNA was extracted from the biopsies of mammary tissue using Trizol Reagent (Invitrogen, Milan, Italy), following the manufacturer's instructions. The concentration of the extracted total RNA was quantified with a spectrophotometer. RNA integrity was evaluated by the observation of 18S and 28S ribosomal bands after electrophoresis on 1% agarose gel in the presence of ethidium bromide. On this criterion, eight samples had intact 18S and 28S RNA while one sample collected at 45 d showed evidence of RNA degradation and was not used for subsequent analysis. In sample analysis, β-actin expression was used as an internal control, confirming the integrity of eight of the nine samples, except for sample collected at 45 d.

ClustalX software was used to align the sequences encoding for bax (Genebank, accession U92569) and bcl-2 (Genebank, accession U92434). Primers were designed with Primer3 Input software and their sequences are listed in Table 1. ClustalX software was also used to align the sequences encoding for rat (Genebank, accession AB04723), human (Genebank, accession XM010246) and mouse (Genebank, accession BC003292) AIF coding sequences (cds). Highly conserved regions of the AIF cds between the examined species were assessed with Genedoc software. In addition, Primer3 Input software was used to design the primer sequences: 5′-GTAGTTTGCCCACAGTTGGTGT-3′

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Table 2. PCR cycles for bovine bcl-2, bax, bcl-2+bax (multiplex), AIF cDNA amplification

cDNA	Denaturation	Annealing	Extension
Bcl-2	94 °C, 30 sec	(58·3 °C, 30 sec) × 40 cycles	72 °C, 1 min
Bax (touchdown)	94 °C, 30 sec	(60 °C, 30 sec) × 6 cycles, (59 °C, 30 sec) × 6 cycles, (58 °C, 30 sec) × 6 cycles, (57 °C, 30 sec) × 6 cycles	72 °C, 1 min
Bcl-2+Bax (multiplex)	94 °C, 30 sec	(56 °C, 30 sec) × 6 cycles, (58 °C, 30 sec) × 10 cycles, (60 °C, 30 sec) × 12 cycles, [60 °C (−0·5 °C/cycles)] × 6 cycles, (57 °C, 30 sec) × 6 cycles, (56 °C, 30 sec) × 6 cycles, (55 °C, 30 sec) × 10 cycles	72 °C, 1 min
AIF	94 °C, 30 sec	(60·3 °C, 30 sec) × 40 cycles	72 °C, 1 min

(AIF for) and 5′-GTAGAAGATGACACCTTTGCCG-3′ (AIF rev). The designed primers amplified a fragment of 203 bp (bases 1541 to 1743) of the human programmed cell death 8 (apoptosis-inducing factor) (PDCD8) cds.

Reverse transcriptase (RT) PCR

RT-PCR reactions were performed using a 'One step' RT-PCR (Invitrogen Milan, Italy) kit. For each reaction, total RNA (1–2 µg) from bovine mammary tissue was retro-transcribed (50 °C for 30 min) and amplified following a PCR protocol. RT-PCR was performed by a MJ thermal cycler (PT-100; MJ Research, Inc. Waltham, MA, USA) and the PCR cycle conditions for different genes are shown in Table 2. PCR reactions carried out with the RNA samples, using this set of primers, gave no amplification product, ruling out the possibility that the observed bands might be due to the presence of contaminating genomic DNA. The RT-PCR product for AIF was purified from agarose with Gel Extraction Kit (Genomed, Berlin-Mahlsdorf, Germany), cloned in pCR II-TOPO cloning vector dual promoter (Invitrogen, Milan, Italy) and sequenced (Primm, Milan, Italy). The sequence was 100% homologous to a Genebank ovine sequence submitted in Genebank (GeneBank access AF529274S1, AF529274S2).

Riboprobe synthesis for AIF

The single-stranded RNA probe was generated using the cDNA obtained from the RT-PCR mentioned above. After cloning, the plasmid was digested with *BamH1*, which cuts downstream of the cloned DNA. T7 polymerase for the antisense probe and SP6 for the sense probe were used to obtain the labelled transcripts incorporating a digoxigenin-11-UTP residue, using the Dig DNA Labelling kit (Boehringer, Mannheim, Germany). Riboprobes were purified and their concentrations evaluated by spectrophotometric analysis.

In situ *hybridization analysis*

The probe generated by RT-PCR analysis was used to hybridize the samples embedded in paraffin and cut at 5 µm. Briefly, the slides were dewaxed with xylene and rehydrated in decreasing concentrations of ethanol through to DEPC-treated water. Samples were rendered permeable in DEPC-treated PBS containing 0·3% Triton X-100 followed by incubation in TE buffer with 20 µg/ml of RNase-free proteinase K. Post-fixation treatment in 4% paraformaldehyde was followed by acetylation, and sections were then incubated in pre-hybridization buffer at 37 °C for 10 min. Hybridization was performed overnight at 42 °C in a humidified chamber in hybridization buffer containing formamide (Sigma, St. Louis MO, USA) with a probe concentration of 10 ng/30 µl per section. Sections were washed in a shaking water bath in 2 × SSC and 1 × SSC at 37 °C and then incubated in NTE buffer pH 8·0 containing 20 µg/ml RNase A to digest the unbound RNA probe. The last washing was performed at 37 °C in 0·1 × SSC. After incubation for 30 min in blocking solution, signal was detected using Dig Nucleic Acid Detection kit (Boehringer, Mannheim, Germany) with NBT/BCIP as colour solution. Slides were incubated overnight in a humid chamber in the dark and then counterstained in 0·1% (w/v) nuclear fast red. Slides were mounted using an aqueous solution and photographed with an Olympus DP10 digital camera.

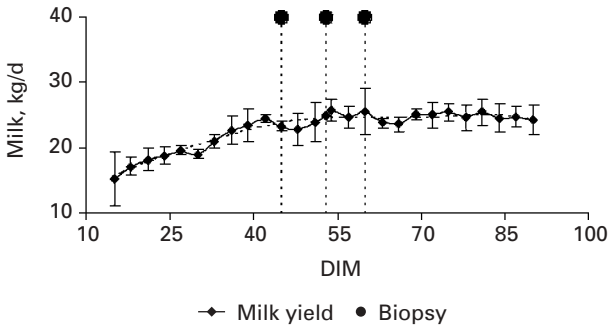

Fig. 1. Daily milk yield in lactating heifers. Yield was measured at 3-d intervals. Values are the mean ± SE for three animals.

Statistical analysis

The ratio of apoptosis to cell proliferation index was first calculated for each heifer (Ratio). The net balance between dying and proliferating cells was estimated using the individual rates of cell proliferation and death at 45, 53 and 60 d after calving. The number of cells in a given day (C_n) was calculated according to Capuco et al. (2001):

$$C_n = C_0 * e^{(0{\cdot}01*P_n - 0{\cdot}01*A_n)*days}$$

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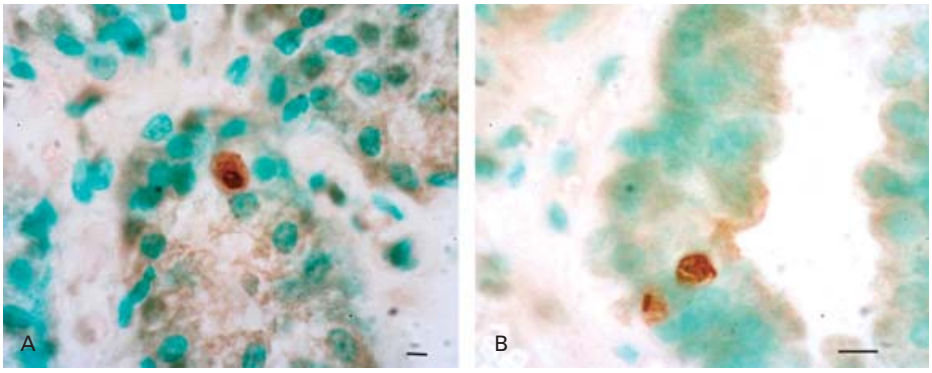

Fig. 2. Identification of apoptotic cells in bovine mammary tissue sections. Representative examples of tissue sections obtained at 53 (A) and 60 (B) days in milk (DIM) are shown. Magnification ×100, scale bar 10 μm.

where C_0 is the number of cells at time 0; C_n is the number of cells at time n>0; P_n is the proliferation index at time n>0; A_n is the apoptotic index at time n>0. For computation, the initial arbitrary value of C_0 was set at 1000 cells. The value for 'days' was set at 6.

Ratio and C_n were correlated with the average milk yield recorded 6 d after biopsies using the Pearson correlation procedure of SPSS (1997). Apoptotic and proliferative indices and the apoptosis to proliferation ratio were analysed by general linear model (SPSS, 1997) with the fixed effect being time of sampling. Homogeneous subsets of means for groups were obtained with the Ryan-Einot-Gabriel-Welsch F test, computed using an alpha level of 0·05.

Results

Cell apoptosis and proliferation

Milk yield measured at 3-d intervals indicated that the animals used in the study were at the peak of lactation at the time measurements were made (Fig. 1). Apoptotic epithelial cells were distributed apparently at random through the alveolar epithelium, as single cells rather than clusters (Fig. 2). No apoptotic bodies were observed, nor was there overt evidence of phagocytic removal of cell remnants. Instead, apoptotic events were evident as TUNEL-positive cells juxtaposed with healthy cells.

The percentage of TUNEL positive cells (apoptotic index) varied greatly between heifers (Table 3) and was not affected by stage of lactation ($P>0·05$). Similarly, the proportion of proliferating cells varied between heifers and did not change significantly as lactation progressed ($P>0·05$). Calculation of the ratio of apoptotic to proliferating cells (Ratio) suggested that, on average, the balance between generation and loss of cells tended towards net cell loss on day 60 (Ratio 1·82 ± 0·32; $P=0·064$), as can also be seen from the individual values for the three heifers.

Correlation analysis (Fig. 3) between average milk yield of each heifer recorded 6 d after biopsy and Ratio was not significant ($r=-0·224$), while a positive relationship with the estimated number of cells was calculated ($r=0·898$; $P<0·01$).

Table 3. Indices of apoptosis and proliferation in mammary tissue of three heifers at peak lactation

Days from calving	Heifer	Index, %†		A/P ratio
		Apoptotic	Proliferation	
45	1	0·56	0·56	1·00
53	1	0·32	0·60	0·53
60	1	0·54	0·40	1·35
45	2	3·08	2·30	1·34
53	2	0·47	1·56	0·30
60	2	0·82	0·34	2·45
45	3	3·94	6·11	0·64
53	3	2·98	2·36	1·26
60	3	3·46	2·09	1·65
Means				
45		2·53	2·99	0·99
53		1·26	1·51	0·70
60		1·61	0·94	1·82
SE				
45		0·20	1·64	0·20
53		0·29	0·51	0·29
60		0·33	0·57	0·33
P of F value		0·635	0·414	0·064

†Apoptotic index: percentage of TUNEL positive cells of total cells counted; Proliferation index: percentage of PCNA positive cells in total cells counted; A/P ratio: ratio of apoptotic to proliferating cells

Immunohistochemistry for bcl-2 and bax

Immunohistochemical analysis was performed on mammary tissue samples to locate bcl-2 and bax proteins. According to Colitti et al. (1999) positive cells were classified in 'strong', 'moderate' and 'light' immunostaining, in comparison with lymphonode cells used for positive control. Moderate immunostaining for bcl-2 protein was observed in all tissue sections, being localized in nuclei, particularly around the inner nuclear membrane. Cytoplasmic bcl-2 staining was granular, or in some cases

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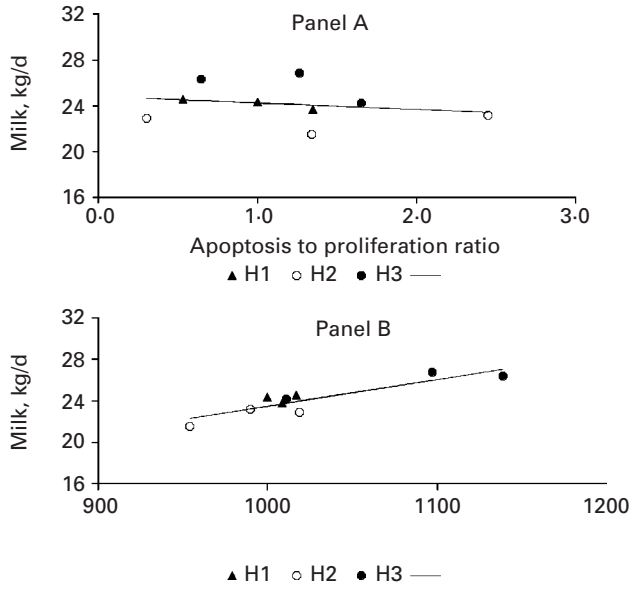

Fig. 3. Graphical appraisal of the relationship between apoptosis to proliferation index (panel A, $r=-0{\cdot}224$; $P>0{\cdot}05$) or estimated cell counts (panel B, $r=0{\cdot}898$; $P<0{\cdot}01$) with average milk yields recorded 6 d after biopsy (▲, Heifer 1; ●, Heifer 2; ○, Heifer 3). Cell counts were estimated from data shown in Table 3 using the following equation: $C_n=C_0*e^{(0{\cdot}01*P_n-0{\cdot}01*A_n)*days}$, where C_0 is the number of cells at time 0; C_n is the number of cells at the time $n>0$; P_n is the proliferation index at time $n>0$; A_n is the apoptotic index at time $n>0$. For the computation, C_0 was arbitrarily set to 1000.

exhibited a network of light staining (results not shown). No immunostaining for bax protein was detected at 45 d post-calving (Fig. 4A). In contrast, progressively stronger positive reactions were detected for bax on day 53 (Fig. 4B) and day 60 (Fig. 4C) in the cytoplasm of cells located in the luminal epithelium.

RT-PCR analysis of bcl-2 family expression

The integrity of mRNA was preliminarily assayed by means of β-actin RT-PCR analysis (Fig. 5D). Expression of bcl-2, bax, and AIF was assessed at 45, 53 and 60 d of lactation. Low expression of bcl-2 mRNA (201 bp) was evident in all samples analysed (Fig. 5A). Expression of bax mRNA (431 bp) was not detected at 45 d, was detected in mammary tissue at 53 d and disappeared at 60 d of lactation (Fig. 5B). Multiplex PCR analysis was also used to compare the relative levels of transcriptional activity for bcl-2 and bax in two tissue samples obtained on day 53 of lactation, and indicated a predominance of bcl-2 expression over bax expression at this stage (Fig. 5E).

Identification of apoptosis-inducing factor (AIF)

Amplification and sequencing of AIF cDNA from bovine mammary tissue revealed a high degree of homology with that of rat (AF262320) and human (O95831). In particular, the bovine AIF partial-cd was highly homologous (89%) between nucleotides 1584–1786 of the rat AIF sequence and nucleotides 1541–1743 that encode for the human PDCD8 (91%). The amino acid sequence of bovine AIF showed still higher similarity between species, with 96% homology for rat AIF (residues 496–562) and 93% with that of the human protein (residues 501–567). AIF is a flavoprotein consisting of an amino-terminal mitochondrial localization sequence (MLS) between amino acids 1 and 102, a spacer sequence of 27 amino acids and a FAD-dependent oxidoreductase domain at the carboxyterminal end of the protein (Daugas et al. 2000). The bovine AIF sequence identified here corresponds to a 67 amino acid sequence in the carboxyterminal domain of the protein.

AIF expression

Expression of bovine mammary AIF mRNA was assessed in a panel of samples that included the three times of sampling. The 203 bp product was not consistently present on days 45 or 53, but was observed in all samples collected at 60 d of lactation (Fig. 5C). However, strong staining indicative of AIF mRNA expression was detected by *in situ* RNA hybridization in alveolar epithelial cells at all three stages of lactation, with the strongest reaction detected on day 53 (Fig. 6). On day 45 of lactation, alveoli expressing AIF were observed juxtaposed with those negative for the protein. Controls performed with the RNA sense probe showed no staining.

Discussion

We investigated the expression of intracellular signals associated with control of apoptosis during a period in ruminant lactation when mammary tissue is fully differentiated and cell turnover is minimal (Wilde & Knight, 1989). By studying this relatively stable phase in a dynamic tissue, after cell number had increased in early lactation and before the net cell loss associated with the natural decline in milk production, we hoped to gain understanding of the subtle modulation of mammary cell turnover that largely determines the persistency of lactation.

Cell loss during lactation appeared to occur without overt changes in the epithelium: apoptotic cell death was confined to individual cells in the alveolar epithelium (Fig. 2). In contrast, even when mice are concurrently pregnant and lactating, as cows are for most of their lactation, weaning precipitates substantial cell turnover and proteolytic remodelling of the extracellular environment (Quarrie et al. 1996).

In a recent paper, we review the evidence that apoptosis is indeed an integral part of bovine mammary tissue's repertoire during lactation, and subject to control by the animal's reproductive status, nutrition and husbandry conditions (Stefanon et al. 2002). For example, cell loss is

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accelerated by infrequent milk removal, as a result of a local, i.e., intra-mammary, stimulation of apoptosis. The mechanisms by which this modulation of apoptosis is exerted during lactation are not understood, possibly because they are subject to a complex and poorly defined hierarchy of systemic and local controls. The extent of mammary cell proliferation and apoptosis varied greatly between individual heifers (Table 3), probably because of their different physiological conditions (Dijkstra et al. 1997), even though they were sampled at the same time interval from calving. Moreover, the balance of cell proliferation and apoptosis is not easy to study because it is a dynamic process but is investigated with single snapshots at a fixed points in time (Hardy & Stark, 2002). Simple mathematical models to evaluate the variation of cell number in mammary gland during lactation have been proposed (Dijkstra et al. 1997; Capuco et al. 2001). These models, as other more general models (Hardy & Stark, 2002), rely on apoptotic and proliferation indices and, though speculative, allow computation of the dynamics of cell population over a known period of time. In the present experiment, we applied the model of Capuco et al. (2001), assuming an initial population of 1000 cells, a value of 6 d, i.e., the time between biopsies, and assuming that durations of apoptosis and proliferation are equal. These authors report that cell apoptosis has an average length of 3 h and consequently the observed apoptotic index needs to be multiplied by 8 to compute the daily rate of cell death. Capuco et al. (2001) based their assumptions on data presented by Bursch et al. (1990), who investigated the length of apoptosis stages in rat liver with histological techniques. It is unlikely, however, that values observed in rat liver apply to mammary cells of cattle, considering species and tissue differences. Bursch et al. (1990) used haematoxylin and eosin to assess the presence of apoptotic bodies, further limiting the adoption of their results to data obtained using TUNEL method. We thus consider that both of the processes have comparable lengths and we just used proliferation and cell death indexes from Table 3. Graphical appraisal (Fig. 3, Panel B) shows a close relationship between the average milk yield recorded and the dynamics of cell population in the mammary gland. Although this correlation has no strict quantitative meaning, it might help in understanding some relevant aspects of mammary biology. The variation of cell numbers for heifers 2 and 3 (H2 and H3) was almost linearly related to milk produced, while for heifer 1 (H1) the small variation of milk yield corresponded well with the small variation in calculated cell number. H2 and H3 had the highest apoptotic and proliferation indexes, which might mean that milk yield is more dependent on cell turn-over than on cell activity, at least in the initial phases of lactation.

We also investigated possible mechanisms regulating cell fate. In other species, and at other stages of lactation, controls on apoptosis are defined in terms of activation of intracellular signalling pathways. Thus, anchorage-dependent mammary cell survival in rodents is characterized by stimulation of pro-apoptotic bcl-2 related proteins (Gilmore et al. 2000), whereas withdrawal of galactopoietic hormones, as at termination of lactation, is associated with induction of insulin-like growth factor binding proteins, which counter the anti-apoptotic effects of IGF-1 and pro-apoptotic bcl-2 family members (Travers et al. 1996; Quarrie et al. 1996). Further, termination of lactation in rodents is associated with reciprocal changes in STAT 3 and STAT 5 signal transduction, favouring STAT 3 actions, which induce tissue remodelling (Chapman et al. 1999). How these various mechanisms integrate should emerge from on-going research in rodents but, even then, it is uncertain whether such information can be extrapolated to ruminants. The lactation cycle of the dairy cow is distinctly different in its responsiveness to stimuli that alter mammary cell number or differentiation. For example, termination of milk secretion is accompanied by limited apoptosis without the extensive tissue remodelling seen in rodents.

To investigate whether putative differences in physiological control of apoptosis between rodents and ruminants extend to the intracellular signalling that determines mammary cell death or survival, we focused on events involving the bcl-2 family of pro- and anti-apoptotic proteins. A principal target of bcl-2 related apoptogenic stimuli appears to be the mitochondrion, and a close link between bcl-2 and bax on the one hand, and permeability transition pores in the outer mitochondrial membrane on the other, may be a key action of these proteins (Crompton, 1999).

It is clear from the present results that, at peak lactation, the cell population in lactating bovine mammary tissue was in a dynamic state, with a low rate of cell proliferation being balanced on day 45 by a similar rate of apoptosis. The rate of cell death at this stage (2·5%) was, as expected, lower that that reported for involuting bovine mammary tissue (4·8%, Wilde et al. 1997). The relatively greater expression of bcl-2 than of bax, indicated by multiplex analysis, is consistent with a modest apoptotic index. There was nevertheless a numerical change in the dynamic balance of the two processes during the course of the study, illustrated by the changing ratio of apoptosis to proliferation in individual animals (Table 3), such that apoptosis predominated on day 60. Subtle changes in the cell population were not accompanied by any marked alteration in bcl-2 or bax expression (Fig. 5A, B). It was clear from immunohistochemical staining, however, that bax protein, which was absent on day 45, had increased on days 53 and 60 and was, at that stage, membrane-associated (Fig. 4C). Expression of bax protein but not bax mRNA on day 60 may be due to greater stability of bax protein compared with its mRNA in dying cells undergoing apoptosis (Heermeier et al. 1996). Detection of bax in lactating bovine mammary tissue contrasts with the situation in lactating rodent tissue, where bax is not normally expressed (Schorr et al. 1999).

The dynamic balance of cell proliferation and apoptosis at peak lactation is supported by the detection of AIF

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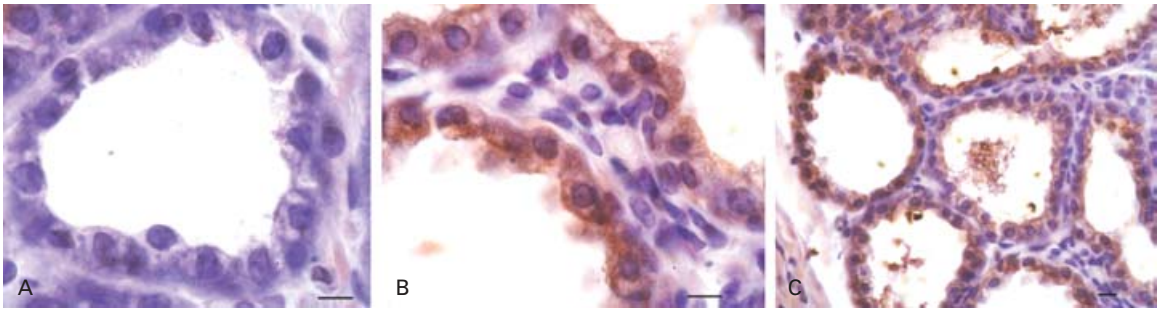

Fig. 4. Immunohistochemical detection of bax in bovine mammary tissue. Representative examples of tissue at 45 (A), 53 (B) and 60 (C) days in milk (DIM) are shown. Note the absence of bax staining on DIM 45, the strong apical staining on DIM 53 and the cytoplasmatic staining on DIM 60. Magnification ×100 (A, B), ×40 (C), scale bar 10 μm.

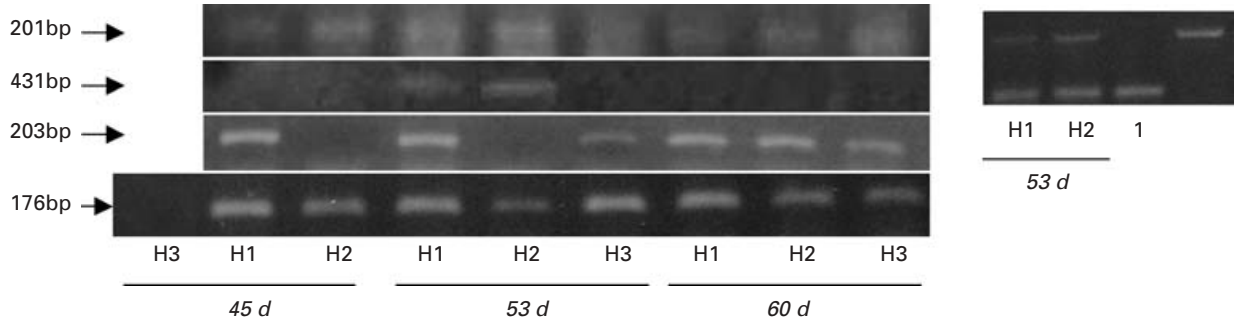

Fig. 5. Detection of bcl-2 (panel A) and bax (panel B), AIF (panel C) and β-actin (panel D) transcripts by RT-PCR. One sample (H3 at DIM 45) was degraded and not included in RT-PCR analyses. H1, Heifer 1; H2, Heifer 2; H3, Heifer 3. Two samples positive for bax mRNA expression were subjected to comparative RT-PCR analysis in which pairs of primers were included in the same PCR reaction (Panel E: Lane 1, bcl-2 mRNA control; Lane 2, bax mRNA control).

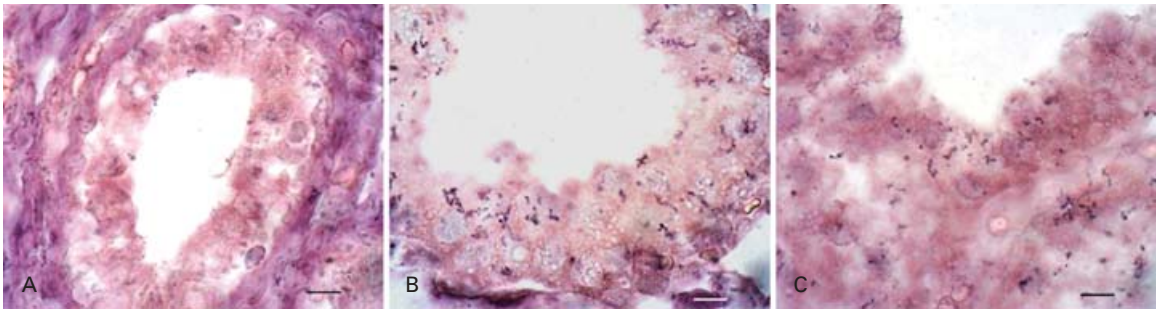

Fig. 6. *In situ* hybridization for AIF transcripts in lactating bovine mammary tissue. Staining was detected in alveolar epithelial cells at all three stages of lactation (45 d, A; 53 d, B; 60 d, D) with lighter reaction at 45 d from calving and strongest at 53 d from calving. Magnification ×100, scale bar 10 μm.

expression at all three stages by RT-PCR and *in situ* hybridization (Figs 5C, 6). AIF expression detected *in situ*, appeared to be associated with cells of the alveolar lumina (Fig. 6), where bax protein predominated (Fig. 4B, C), confirming that the apoptotic signalling was likely to elicit changes in the population of milk-producing cells, and suggesting that the two signals may be causally related. AIF, which has not previously been studied in bovine mammary tissue, responds in other tissues to mitochondrial bax : bcl-2 heterodimerization by translocating from the mitochondrion to the nucleus. In human tissues, AIF translocation precedes cytochrome c release and caspase activation in the cascade of events that precipitates apoptosis (Ferr et al. 2000), and the translocation is blocked in cells over-expressing bcl-2 (Daugas et al. 2000). Our results showed that bax protein expression was stronger at 60 d than at 45 d of lactation, while bcl-2 protein remained relatively constant (data not shown). Assuming the AIF message is translated, an increase in bax : bcl-2 heterodimerization with stage of lactation would then promote

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AIF translocation and contribute to a shift in the dynamic balance in the cell population from proliferation towards apoptosis, as we found in this study.

AIF expression alone is no indicator of apoptogenic signalling, since it is expressed ubiquitously in healthy human tissues, and is detectable by immunohistochemistry in normal epithelial cells of the kidney and duodenum (Daugas et al. 2000). More experiments are therefore needed to determine whether up-regulation of mammary AIF expression is associated with nuclear translocation and an apoptotic stimulus therefrom.

The present study demonstrates the expression of members of the bcl-2 family and AIF in bovine mammary tissue around peak lactation. The results suggest differences between rodent and ruminant mammary tissue in the roles played by these signalling molecules, notably bax (Schorr et al. 1999). Furthermore, they illustrate a dynamic balance in the intracellular signalling associated with apoptosis in bovine mammary tissue consistent with modulation of the mammary cell population, a factor that in turn is likely to influence lactation persistency in the dairy cow (Li et al. 1999).

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