Measurement of cell death by *in situ* end labelling of ruminant mammary gland tissue

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ABSTRACT

Apoptosis was examined by *in situ* end labelling (ISEL) of DNA in ewe and cow mammary sections representing the cycle of mammary development, lactation and regression. Labelled nuclei, identifying apoptotic dying cells, were found in tissues from all developmental stages. Generally, apoptosis was low in tissue from virgin animals, moderate in 3 and low in 2 week udders from prepartum ewes and mid pregnant cows, moderate to high in udders from 1 week post partum ewe udders, moderate to low in lactating ewes and cows and slightly higher in involuting ewe and cow udders. Apoptosis was also generally higher in alveoli in stasis than in lactating alveoli. A dramatic finding was that there was intense labelling with both ^{35}S and DIG in the lumenal contents of some alveoli in stasis/involution particularly with animals in later lactation. This suggested that there was a significant amount of ragged DNA ends in the lumen of these alveoli.

Keywords: ovine; bovine; mammary; apoptosis; *in situ* end labelling.

INTRODUCTION

Previous studies in this lab have shown that some alveoli in the lactating gland are quiescent (Molenaar *et al.*, 1994) and are easily identified histologically due to the presence of large vesicles engorging the secretory cells and in most lumena. These vesicle engorged alveoli (VeA) alveoli can be grouped into two categories, one where an abrupt transition of expression of α-lactalbumin and casein versus lactoferrin occurs at the basement membrane between lactating and non-lactating alveoli and another where a gradient in the of transition gene expression occurs which is nearly one alveolus wide. Alveoli in the second category tend to contain elevated numbers of leucocytes and sloughed epithelial cells. This suggests that some epithelial cells in these alveoli may be dying.

Apoptosis, also known as active or programmed cell death, is a normal and essential process by which cells are instructed to die in a regulated manner. Apoptosis provides a mechanism for the removal of superfluous cells and a means of shaping structures during morphogenesis (Ellis *et al.*, 1991). It involves a controlled breaking up of the cellular DNA which can be visualized as a laddering pattern when the DNA is electrophoresed through agarose gels (Naora and Naora, 1995) or by a variety of techniques such as *in situ* end labelling (ISEL) of DNA (Quarrie *et al.*, 1995), *in situ* nick translation (Gold *et al.*, 1993) and terminal transferase mediated dUTP-biotin nick end labelling (Gavrielli *et al.*, 1992). When the cells die they are phagocytosed by leucocytes or neighbouring cells (Martin *et al.*, 1994).

Apoptosis has been shown to occur in the mammary gland (Gavrielli *et al*., 1992; Atwood *et al.*, 1995; Bielke *et al.*, 1995; Quarrie *et al*., 1995). Most studies have concentrated on the involution phase in mice and have given differing results. An early study of the first 6 days after weaning in mice demonstrated only occasional nuclear labelling (Gavrielli *et al*., 1992). In mice, a peak in the intensity of the DNA laddering was observed to occur between 3 and 5 days involution (Atwood *et al.*, 1995). Using an end labelling technique, positive cells were observed by 48 hours post weaning with apparently all of the secretory epithelial cells being labelled at 3 and 4 days post weaning (Bielke *et al.*, 1995). Another end labelling study of lactating and involuting glands demonstrated that numbers of labelled nuclei increased from <0.2% of the alveolar cells in lactating glands to 1.7% and 4.5% at 2-3 and 4 days after litter removal respectively (Quarrie *et al*., 1995).

We report here some preliminary studies which attempt to shed some light on the fate of these alveoli in milk stasis by examining apoptosis in mammary epithelial cells in ewes and cows during lactation and also throughout the cycle of mammary development.

MATERIALS AND METHODS

Tissues

Mammary samples were taken from ewes that were virgin (n=2), ~3 and ~2 weeks prepartum (n=1 and n=2 respectively), 1 week postpartum (n=3), 10 weeks lactating (n=2) and ewes that were weaned at 10 weeks lactation and sampled at 2, 4, 10 and 40 days of involution (n=1 each stage). Similarly samples were taken from cows whose offspring were 65% (n=1), and 90% (n=1) progressed through gestation, from lactating dairy cows with one involuting quarter (n=1) and beef cows in involution (n=3). Tissues were formalin fixed, processed and embedded in wax and sectioned as previously described (Molenaar *et al.*, 1991).

In Situ End Labelling (ISEL)

Two informative sections from a 10 week lactating ewe and one from each other animal tested were selected for examination. ISEL was performed using a modified

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method of Ansari *et al.*, (1993). The sections were dewaxed, treated with proteinase K and dried as previously described (Molenaar *et al.*, 1991). ISEL was performed directly on the slide in a 50 µl volume using the Klenow fragment of DNA polymerase to incorporate either ^{35}S dATP or, digoxigenin (DIG) label, into fragmented or damaged DNA. 100 µl of reaction contained 24 µl of 5x reverse transcription buffer (Promega, USA), 2 µl of each 1 mM dGTP, dCTP and dTTP, 2 µl of bovine serum albumin (Molecular Biology grade, Boehringer, Germany) 3 µl of ß-mercaptoethanol (diluted 1/10), 70 µl Milli Q filtered water, 2 µl Klenow (Boehringer) and 1 µl of either ^{35}S dATP (Amersham, England) or digoxigenin d11-UTP (DIG) (Boehringer). For controls the Klenow was omitted from the reaction. The reactions were allowed to proceed for 2-3 hours at 37'C in a humidified chamber then washed in several changes of 0.2x Sodium Citrate buffer. The slides were washed and the ^{35}S was label detected by autoradiography, initially with XAR5 film (Kodak, USA) with 30 minutes exposure, then K5 Emulsion (Ilford, England), for 2 days, as previously described (Molenaar *et al.*, 1991). The DIG label was detected using the DIG immunohistochemical detection kit using alkaline phosphatase (Boehringer, 1993). The alkaline phosphatase reaction was was allowed to proceed for 1-3 hours. Numbers of labelled nuclei in mammary tissue were counted during examination by light microscopy and expressed as the number of nuclei per mm^2 of specific tissue types.

RESULTS

Labelled nuclei were found in tissues from all developmental stages but the frequency of labelled nuclei varied from other studies in mice. Detection with DIG was more sensitive than ^{35}S but if the detection reaction was allowed to proceed too long, non specific detection of DNA breaks due to normal processing of the tissues could occur in all nuclei. The ^{35}S label was more stoichiometric (Bielke *et al.*, 1995) than DIG but a problem occurred with its adherence to corpora amylacea bodies (Arnold and Weber, 1977) in lactating tissues. These factors made scoring difficult. The two labels did, however, give similar results (Figures 1 and 2). in the relative number of labelled cells at different developmental stages, but with some variations. Figure 1 and 2 express labelled nuclei as numbers per mm^2 of all tissue, lactating tissue and VeA. Generally, apoptosis was low in tissue from virgin animals, moderate in 3 (Figure 3A, 3B is a negative control) and low in 2 week (Figure 3C) udders from prepartum ewes and mid pregnant cows, and moderate to high in udders from 1 week post partum ewe udders (Figure 3D, 3E is a negative control, and 3F). Numbers fell slightly in 10 week lactating tissues (Figure 3G) compared to 1 week lactating tissues and increased slightly in tissues in involution (Figure 3H, 3I). Except for one ewe udder at 1 week lactation there was more labelling in tissue in stasis than in lactating tissue although the intense signal also made scoring difficult (see below). The areas lacking α-lactalbumin mRNA expression shown in figure 3J indicate lobules that were in stasis. Note that these areas correspond to the diffuse grey labelled areas in figure 3I. Extensive apoptosis in udders from involuting animals, as has been reported in mice (Bielke *et al.*, 1995), was not observed.

A dramatic finding was that of intense labelling with both ^{35}S and DIG in the lumenal contents of some alveoli in stasis, particularly in udders that were later in lactation (note the differing intensities of labelling in the lobules of static alveoli shown (Figure 3G) and in involution (Figure 3H). This intense reaction occurred in the alveoli which had been shown previously to have high expression of

FIGURE 1: Measurement of labelled nuclei per area of tissue in udders and their lactating versus non lactating components from ewes and cows in various stages of development using *in situ* end labelling with ^{35}SdATP. The numbers in brackets refer to the animal identity in each category.

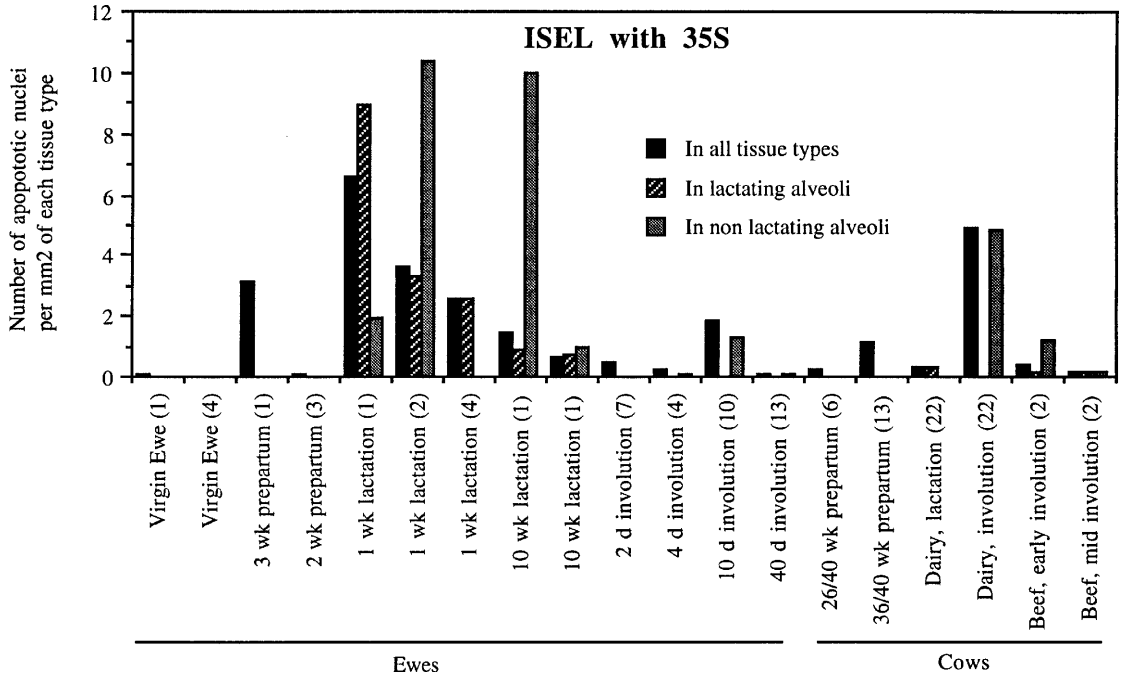
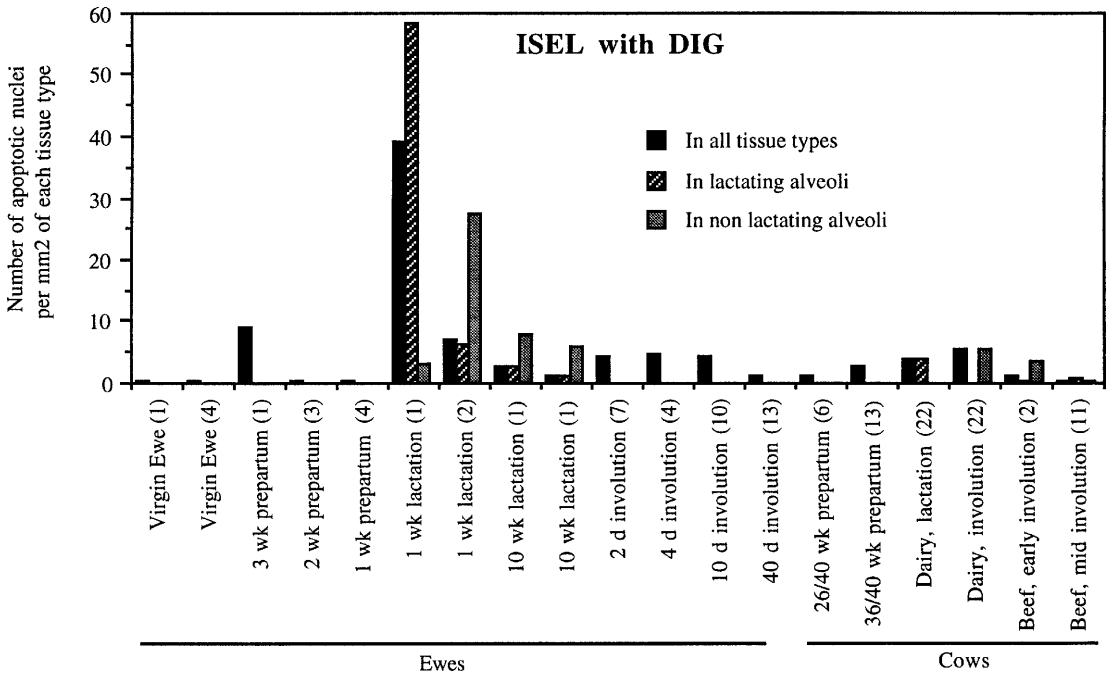


FIGURE 2: Measurement of labelled nuclei per area of tissue in udders and their lactating versus non lactating components from ewes and cows in various stages of development using *in situ* end labelling with digoxigenin-11-dUTP (DIG). The animal identities are the same as for figure 1.



lactoferrin mRNA (data not shown). In a 10 week lactating udder, a more intense reaction (Figure 3L) was seen in VeA that contained leucocytes and were adjacent to the gradient in the transition of α-lactalbumin mRNA expression (Figure 3K) than those that did not contain leucocytes (Figure 3M) and were adjacent to the abrupt transition of α-lactalbumin mRNA expression (Figure 3N). Interestingly, the intense labelling of the alveolar contents of VeA was not observed (Figure 3E) in an udder from a 1 week lactating ewe that had been shown to exhibit the gradient of α-lactalbumin mRNA expression (data not shown).

DISCUSSION

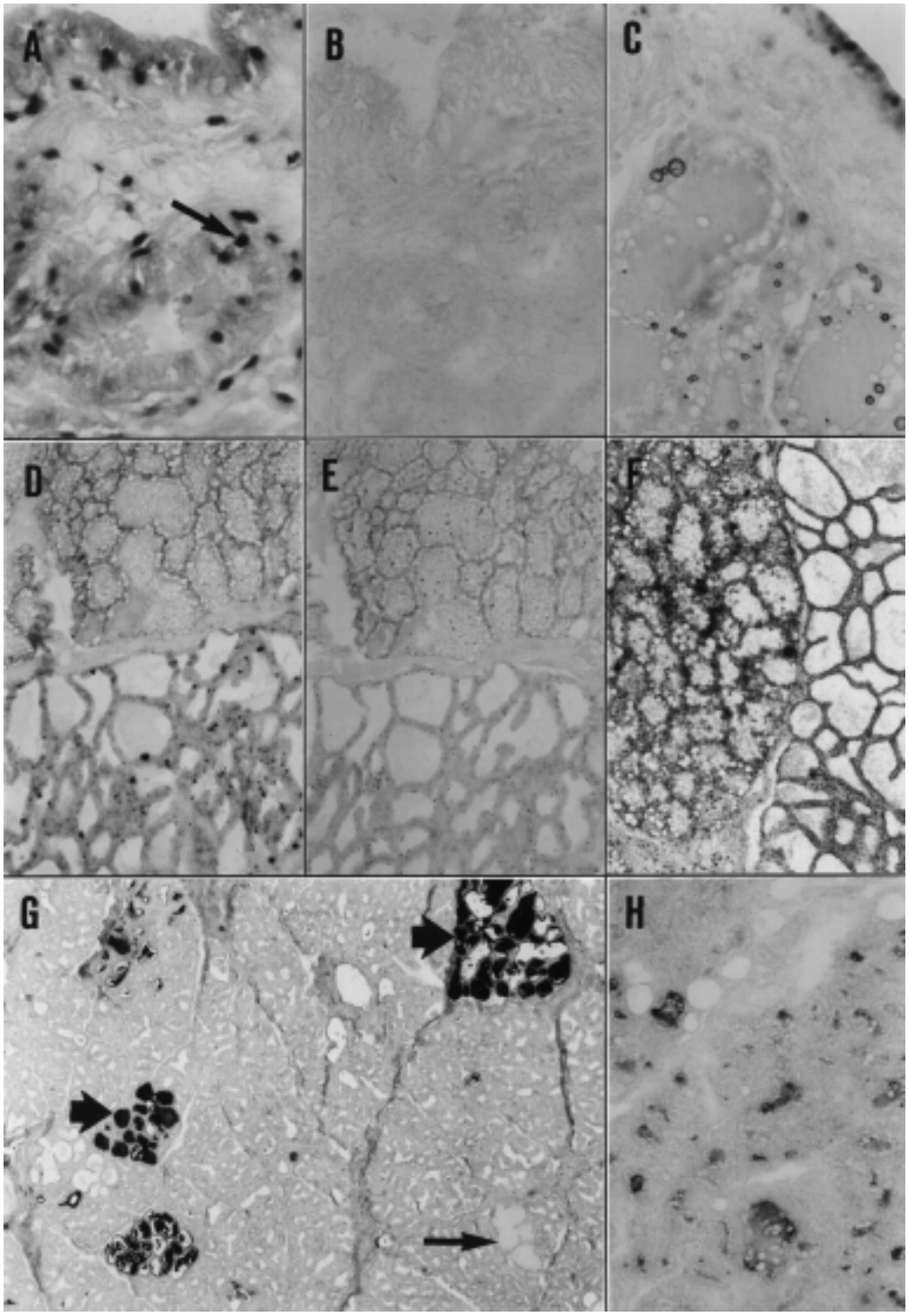
As tissue remodelling involves both cell proliferation and cell death (Boudreau *et al.*, 1995), The relative numbers of labelled nuclei found in glands from pregnant and involuting animals could be predicted when the developmental changes were considered. Although the small sample size reflects the preliminary nature of the study, the very low number of apoptotic cells in the udders both immediately before parturition, and at weaning suggests that under these conditions the whole udder may go into some kind of inactive state. The high numbers of labelled nuclei in the lactating cells of one, 1 week lactating ewe was unexpected. This animal's udder also contained a relatively high proportion of lobules containing VeA. It is possible that insufficient demand for milk by the neonate could account for the increased numbers of apoptotic nuclei in lactating tissue. The observation that numbers of labelled nuclei in mid involuting tissues was only slightly increased, that is the massive cell death that occurs in rodents at involution did not occur, supports studies showing that involution in ruminants is a more gradual process (Holst *et al.*, 1987; Guenette *et al.*, 1994) than in rodents. Furthermore, it is most interesting that even late involution tissue, only a few of the cells remaining fixed to the basement membrane were labelled. This suggests that the alveolus as a whole may exist in stasis for an extended period but may still be capable of regaining secretory activity at any time.

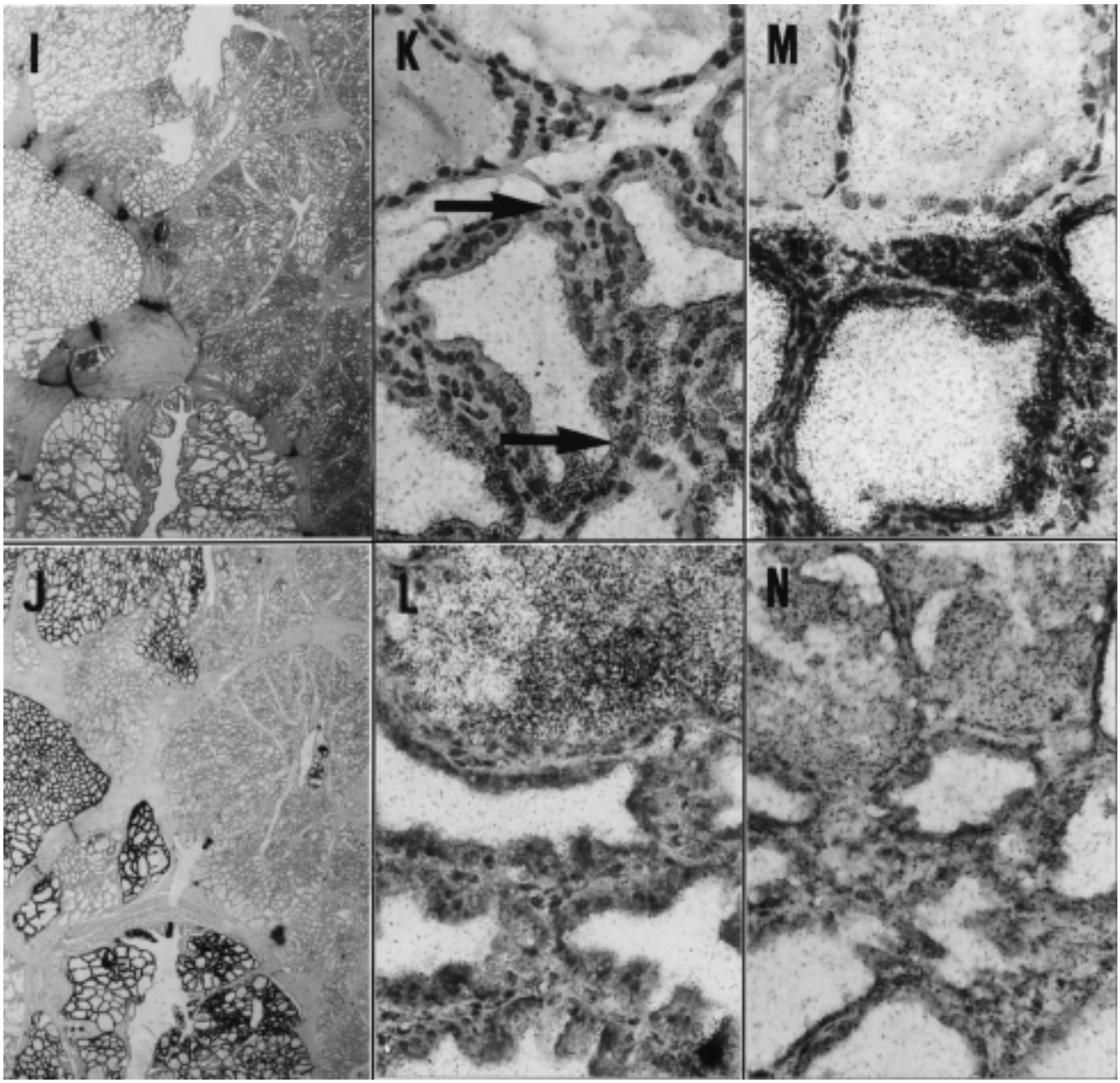
The intense labelling observed in the alveolar lumen of alveoli in stasis was unexpected. The most probable explanation for this intense labelling is that there was a significant amount of ragged DNA ends in the lumen. This DNA could have originated from lysed bacteria but they were not evident on Haematoxylin and Eosin or giemsa staining. The lysis of even a few mammary epithelial cells in the lumena would liberate a large amount of ragged DNA. Lysed mammary epithelial cells are the most likely source of DNA present and the intensity of staining could be related to the length of time the alveoli had been in stasis. This is supported by a similar observation in alveoli from udders at 10 days of involution. To reiterate, the fact that there were few labelled nuclei in cells adhered to the alveolar basement membrane indicates only that a few cells of these alveoli were dying, not all the secretory cells of the whole alveolus. To account for the disparity between the intense labelling of lumena in VeA adjacent to the gradient of α-lactalbumin expression in 10 week lactating ewes, and the non labelling of the lumena in static alveoli of 1 week lactating ewe exhibiting the same gradient, despite the presence of labelled nuclei attached to the basement membrane, it is necessary to propose that this animal had been sampled before the labelled cells had become detached and lysed.

Further work is needed to identify the source of free DNA in the lumen of many VeA. If the intensity of

FIGURE 3: A; ISEL with DIG on an udder from a 3 week prepartum ewe, the arrow indicates a labelled nucleus. B; Negative control for A. C; ISEL with DIG on an udder from a 2 week prepartum ewe. D. ISEL with DIG on an udder which contained a large proportion of vesicle engorged alveoli (VeA) (shown in the upper portion of the micrograph) from a 1 week lactating ewe. VeA contain large visible vesicles in the cells and lumen. E; Negative control for D. F; ISEL with ^{35}SdATP on an udder from a 1 week lactating ewe which contained a small proportion of VeA (left side of the micrograph) and which had a gradient of α-lactalbumin expression in adjacent lactating alveoli. G; ISEL with DIG on an udder from a 10 week lactating ewe. The small long arrow indicates a lobule of VeA with unlabelled lumena. The short wide arrows indicate two lobules of VeA with different labelling intensities. H; ISEL with DIG on an udder from a 10 week post partum ewe after 10 days of non lactation. Most lumena are labelled. I; ISEL with ^{35}SdATP on an udder from an early involution cow. The grey label is shown predominantly on the right side of the micrograph. J; *In situ* hybridisation with ^{35}S-UTP to α-lactalbumin mRNA in a serial section of I. α-Lactalbumin mRNA was predominantly absent in the tissue shown on the right side of the micrograph. K; *In situ* hybridisation to α-lactalbumin mRNA performed on an udder from a 10 week lactating ewe and showing a gradient of expression (located between the arrows) adjacent to a lobule of VeA. The VeA are in the upper part of the micrographs for K-N. L; ISEL with ^{35}SdATP on a near serial section of K and showing the same lobule. M; The same section as K showing another VeA lobule but one with an abrupt transition of α-lactalbumin expression adjacent to it. N; ISEL with ^{35}SdATP on the same section as L and showing the same lobule as in M.

A-C, and K-M are magnified 400x. D-F are magnified 100x. G and H are magnified 40x. I and J magnified 10x. A-H except F are not counterstained. F and I-N are counterstained with Haematoxylin and Eosin.





staining could be calibrated, it may be possible to use the ISEL technique to roughly estimate the length of time alveoli have been in stasis, assuming that the DNA was not of bacterial origin.

Several conclusions can be made from these results. Firstly, that apoptosis is a normal event which occurs at most stages of mammary development. Secondly, that the profile of apoptosis does not closely match that of the rodent. Thirdly, that cells that are in stasis with respect to milk synthesis are not necessarily undergoing involution.

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