MAMMARY APOPTOSIS

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A thesis submitted to the University of Glasgow in accordance with requirements for the degree of Doctor of Philosophy in the Faculty of Science.

Hannah Research Institute, Ayr

March 1996.

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LIST OF ABBREVIATIONS

AGS	-anti growth hormone serum
Br	-bromocriptine
DAB	-diaminobenzidene
DAPI	-diaminophenylindole
cortisol	-hydrocortisone-21-acetate
EHS	-englebreth holm swarm
FIL	-feedback inhibitor of lactation
GH	-growth hormone
h	-hour or hours
H+E	-haematoxylin and eosin
IGF	-insulin like growth factor
IGFBP	-insulin like growth factor binding protein
IL	-interleukin
ISEL	-*in situ* end labelling
P	-probability
RMYQ	-relative milk yield quotient
SDS	-sodium dodecyl sulphate
SEM	-standard error of the mean
TIMP	-tissue inhibitor of metalloproteinase
Tris	-tris(hydroxymethyl)aminomethane
WAP	-whey acidic protein
ABC	-avidin biotin complex
OCT	Optimum cutting temperature

ACKNOWLEDGMENTS

I wish firstly to thank Drs. Colin Wilde and Caroline Addey for supervising this project, and for giving me so much advice and encouragement throughout my three years at the Hannah Research Institute. I also wish to extend this "thank-you" to all members of the Director's group for making my stay an enjoyable and memorable one. In particular, I would like to thank Dave Blatchford for firstly showing me the mouse mammary cell culture techniques and for helping me with so many of the culture experiments. I also wish to thank Dr. Peter Bird, who, as a visiting worker to the Director's group, was so interested in my project and helped me develop the [^{32}P] nick end labelling method for genomic DNA. In addition, I will not forget the help, enthusiasm and encouragement of Dr. Mike Barber, without whom my study of mouse mammary apoptosis would be nowhere near as comprehensive or as interesting.

As for the photography, I wish to thank both Derek Cooney of the Hannah Research Institute and Trevor Graham at the University of Glasgow. From outside the Institute I also wish to thank Dr. Bijan Ansari, who gave me valuable advice on ISEL cell staining techniques and to thank those I have not met who gave me cDNA probes for use in these studies.

LIST OF PUBLICATIONS

The results presented in his thesis have been published in part in the following journals.

Quarrie, L.H., Addey, C.V.P. & Wilde, C.J. (1994). Local regulation of mammary apoptosis in the lactating goat, *Biochem. Soc. Trans.*, **22,** 178S.

Quarrie, L.H., Addey, C.V.P. & Wilde, C.J. (1995). Local regulation of mammary apoptosis by milk stasis, In *intracellular signalling in the mammary gland,* (eds. Wilde, C.J., Peaker, M. & Knight, C.H.), 95-96, Plenum Press, London.

Quarrie, L.H., Addey, C.V.P. & Wilde, C.J. (1995). Apoptosis in lactating and involuting mouse mammary tissue demonstrated by nick-end DNA labelling, *Cell Tissue Res.*, **281,** 413-419.

Tonner, E., Quarrie, L.H., Travers, M., Logan, A., Beattie, J., Wilde, C.J. & Flint, D.J. (1995). Does IGF- binding protein (IGFBP) present in involuting rat mammary gland stimulate apoptosis? *Proceedings of the third international symposium on IGF binding proteins,* Tuebingen, October, 1995.

Quarrie, L.H., Tonner, E., Blatchford, D.R., Flint, D.J. & Wilde, C.J. (1996). Regulation of apoptosis in cultured mammary epithelial cells, In *Animal cell technology: basic and applied aspects,* Vol 8, (ed. Matsushita, T.), Kluwer Press, Dordrecht, in press.

Travers, M.T., Barber, M.C., Tonner, E., Quarrie, L.H., Wilde, C.J. & Flint, D.J. (1996). The role of prolactin and growth hormone in the regulation of casein gene expression and mammary cell survival; relationship to milk synthesis and secretion, *Endocrinology,* in press.

Quarrie, L.H., Addey, C.V.P. & Wilde, C.J. (Programmed cell death during mammary tissue involution induced by weaning, litter removal and milk stasis, *J. Cell Physiol.*, In revision.

DECLARATION

All data were collected by myself, with the exceptions of animal experimentation performed by Dr. D.Flint and M. Gardiner in Chapter 4 and the IGFBP ligand blot and IGFBP Northern blot performed by E. Tonner and Dr. M. Travers, respectively in Chapter 6. With these exceptions, I declare that the work contained in this thesis is my own, undertaken under the supervision and guidance of Dr. C.J. Wilde and Dr. C.V.P. Addey. No part of this work has been submitted for consideration for any other degree or award.

Lynda H. Quarrie.

ABSTRACT

This thesis examines the importance of mammary apoptosis during lactation and involution and investigates some of the factors which influence apoptotic rates in the mammary gland.

The extent of apoptosis in mammary tissue was assessed using [^{32}P] dCTP nick-end labelled DNA to visualise DNA laddering. Changes in DNA laddering were compared with histological techniques to determine general tissue morphology, chromatin changes and presence of apoptotic bodies and localisation of dying cells (ISEL). Where possible these measurements were combined with gene expression studies to determine changes in expression of proteases, inhibitors of proteases, p53 and bax.

As shown previously, apoptosis is important for cell removal in rodents during natural weaning and after litter removal at peak lactation. In both situations apoptosis followed a similar timecourse, however, after litter removal in declining lactation the increase in apoptosis was more rapid. Apoptosis also appeared to be essential between lactation cycles even when the interval between lactation cycles was reduced to a minimum by mating at *post partum* oestrus. DNA laddering was detected at peak lactation of mice, rats and goats, suggesting that cell turnover is a physiological process in lactating tissue.

This study also investigated the regulation of mammary apoptosis. The effects of hormonal status of lactating rats and milk stasis in mice and goats were investigated. Prolactin, acting as a cell survival factor, was shown to influence apoptosis. Growth hormone action was more subtle, and was achieved through a different mechanism to

that of prolactin. A local mechanism for regulating mammary apoptosis was demonstrated in mice where unilateral milk stasis induced apoptosis at levels similar to litter removal. Apoptosis was also induced in goats by a local control mechanism since unilateral cessation of milking increased apoptosis to a greater degree in unmilked glands compared to milked glands.

Finally, possible mechanisms of local and systemic control were studied during lumen formation when mammary cells were cultured on extracellular matrix, *in vitro*. After several days on extracellular matrix solid clumps of epithelial cells form hollow spherical structures. Cells on the periphery of the cell clump survive, and only these have access to laminin, (a component of the extracellular matrix). Cells in the centre of the clumps die by apoptosis, and this produces luminal spaces within the structures. An IGFBP is also elevated at the time of apoptotic cell removal, and may be involved in blocking IGF-1 activity in cells that are to die.

In conclusion, this study demonstrates the widespread importance of apoptosis in the mammary gland, the local and systemic regulation of the process and some clues as to the mechanism of apoptosis in mammary cells.

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 Discovery of apoptotic cell death

A new and orderly sequence of events during cell death was described by Kerr (1965), while investigating histochemical changes during hepatic ischaemia in the rat. It was discovered that during parenchymal regression, individual hepatocytes underwent a series of histological changes which differed from those of necrotic cells (Kerr, 1965). The hepatocytes were continually converted into small round cytoplasmic masses, some of which contained fragments of pycnotic chromatin. The lysosomes in these small round masses stained discretely, suggesting they were intact. Importantly, apparently identical rounded cytoplasmic masses were detected in small numbers in the livers of healthy rats. This process was referred to initially as "shrinkage necrosis" (Kerr, 1971). However the use of the term necrosis for a phenomenon that occurred under physiological conditions seemed undesirable, so the process was renamed *apoptosis* (a Greek word used to describe the shedding of leaves from a tree or petals from flowers; Kerr et al., 1972).

The same sequence of apoptotic events has been detected in a great variety of animal tissues. An apparently similar process, at least as far as nuclear changes are concerned, occurs in normal plants (Eleftheriou, 1986). This suggests that apoptosis is a process which originated early in biological evolution.

1.2 Types of cell death

1.2.1 Characteristics of programmed cell death

The morphological changes observed in cells undergoing programmed cell death include controlled condensation of the cell, shrinkage of cytoplasm, condensation of chromatin and absence of inflammatory reaction. This showed this type of cell death resembled apoptosis more than necrosis. Presently, programmed cell death is considered to be an apoptotic type of cell death. Death is referred to as programmed or apoptotic depending upon whether the death is planned in a developmental sequence (programmed cell death) or is a potential solution to an inadvertant but dangerous situation (apoptosis). However, there is considerable overlap between the two processes and it is often not clear if death is apoptotic or programmed. Therefore the two terms are often interchangable.

1.2.2 Characteristics of necrosis

Events during necrotic cell death have been reviewed by Walker et al. (1988). Until the 1980's cell death was regarded solely as a necrotic process, i.e. a relatively uncontrolled progressive degradation of cell structure that occurs after death, caused by severe injurious changes in environmental conditions. For example, necrotic death occurred after exposure to hyperthermia, hypoxia, ischemia, complement attack or metabolic poisons (Schanne et al., 1979; Trump et al., 1981; Laiho et al., 1983).

Necrotic cells often loose specialised surface structures such as microvilli and develop surface blebs (Ginn et al., 1969), and also show mild swelling of mitocondria and cytoplasm, dilation of endoplasmic reticulum (ER), depletion of glycogen, dissociation of ribosomes, dispersal of polysomes and irregular clumping of chromatin

around the nuclear membrane. The plasma, organelle and nuclear membranes become more permeable, and fluid moves more freely across these membranes causing cells to swell. Further mitochondrial swelling occurs. Mitochondria now appear flocculent or contain densely granular matrix. Such an appearance is regarded as being the earliest reliable ultrastructural marker of necrosis (Jennings and Reimer, 1981). Lysosomal enzymes are released, resulting in destruction of cell organelles, chromatin disappears to leave ghost nuclei (karyolysis) and the cells membranes are no longer intact (Hawkins et al., 1972; Kloner et al., 1974). Breakdown in cellular components is coincident with the release of free fatty acids, free amino acids and inorganic phosphates from the necrotic cells (Trump et al., 1981; 1984). DNA exposed by proteolytic digestion of histones, is cleaved by lysosomal deoxyribonuclease into fragments displaying a continuous spectrum of sizes (Afanasev et al., 1986; Duvall and Wyllie, 1986).

Typically, groups of contiguous cells are affected and an inflammatory reaction develops in adjacent viable tissue. The necrotic remains are ultimately removed by mononuclear phagocytes.

1.2.3 Characteristics of apoptosis

Apoptotic cell death typically occurs in scattered cells and progresses so rapidly that it is often difficult to observe. The first detectable change is the compaction of chromatin into crescent shaped areas or dense masses that abut onto the nuclear envelope. The cytoplasm of a cell condenses. Nuclear DNA undergoes characteristic changes. Double strand cleavage occurs at the linker regions between nucleosomes to produce fragments which are multiples of 185 base pairs (Wyllie, 1980; Arends et al.,

1990). DNA degradation is specific since there is no apparent proteolysis of histones or other nuclear proteins (Wyllie et al., 1986) and the nuclear matrix of apoptotic cells remains unaltered (Arends et al., 1990). The near-universal presence of internucleosomal cleavage in apoptotic cells suggests that there may be a common mechanism by which apoptosis occurs in different cell types, and also that DNA fragmentation is an important part of the cell death mechanism. In exceptional circumstances key morphological features of apoptosis may be observed in the absence of internucleosomal DNA fragmentation (Collins et al., 1992), or DNA fragmentation may only be barely detectable at advanced stages of apoptosis (Zakeri et al., 1993).

To date, several candidate molecules for the apoptotic endodeoxyribonuclease responsible for internucleosomal DNA cleavage have been characterised or detected in various tissues or cell lines. They include a 130 kDa anionic protein with DNase activity (Arends et al., 1990), NUC-18 and a calcium dependent DNase (Alnemri and Litwack, 1990). DNase 1 has also been implicated (Pietsch et al., 1992: Ucker et al., 1992), as have DNase II and NUC 1 (Barry and Eastman, 1993). Several studies have shown the endonuclease responsible for apoptotic cleavage of DNA to be calcium and magnesium dependent and inhibited by zinc (Cohen and Duke, 1984). The endonuclease responsible for apoptotic DNA cleavage has not yet been fully purified and characterised and so no general concensus of opinion exists as to its identity (Giadio and Cidlowski, 1991; reviewed by Schwartzman and Cidlowski, 1993).

As the compaction of chromatin continues, electron microscopic studies show the nuclear and cellular boundaries becoming more convoluted. Within the nuclei, chromatin usually becomed distributed as periperal crescents. At this stage vesicles are then extruded from the main body of the cell and are known as apoptotic bodies.

These vesicles are membrane destined and of different sizes containing some intact organelles. The apoptotic bodies may or may not contain fragments of chromatin. When the apoptotic bodies are released from the surface of single layer epithelium systems they are either taken up rapidly by adjacent cells and degraded by lysosomes, or are engulfed by neighbouring macrophages (Savill et al., 1993). The swift and efficient engulfment of the dead cell ensures macromolecules are not released from the dying cells provoking an immune response. Surrounding cells will close ranks so cell deletion occurs without disrupting tissue architecture. There is no inflammation and so the process is histologically remarkably inconspicuous.

1.3 Regulation of apoptosis

In some situations the presence of a protein synthesis inhibitor will prevent apoptosis, suggesting protein synthesis is a requirement of apoptosis (Cohen and Duke, 1984; Wyllie et al., 1984; Compton et al., 1988). In other situations, protein synthesis inhibitors do not prevent apoptosis (Waring, 1990; McCall and Cohen, 1991) suggesting all the proteins necessary for apoptosis are already present. Indeed in some situations protein synthesis inhibitors have been reported to induce apoptosis (Martin, 1993). These conflicting results suggest that cells may be in different stages of preparedness for apoptosis (Ledda-Columbano et al., 1992; Martin, 1993) or that protein synthesis may be essential for initiation only by certain types of stimuli and it is not an absolute requirement for execution of the process (Zacharchuck et al., 1990; Iseki et al., 1991). Alternatively it is possible that protein and RNA synthesis is required for the regulation of apoptosis rather than for synthesis of components of the

programme itself.

1.4 Mechanism of apoptosis

The mechanism of apoptosis has not been elucidated. However, changes in the expression of certain genes are known to be a feature of apoptotic deaths. Although tissue specific gene expression may contribute to the extent of apoptosis in particular tissues, some changes in gene expression are universal and these form the general mechanism of apoptosis.

It is not yet clear if the events during apoptosis are directly coupled, so that one event triggers the next, or whether individual steps in the sequence are controlled independently by a cytoplasmic control system, as occurs during cell cycle regulation. Indeed, there are similarities between the mechanisms controlling the cell cycle and apoptosis. Perhaps the most important genetic changes observed during apoptosis are changes in the pattern of expression of proto-oncogenes and anti-oncogenes that control the cell cycle (Columbel et al., 1992). From this evidence it appears that the cell cycle and apoptosis share a common regulatory pathway that diverges at some point where a cell either divides or dies.

Of the many oncogenes that influence cell cycle control, to date, the roles of *bcl-2* and *p53* are perhaps the most interesting with respect to apoptosis.

1.4.1 Regulatory role of *bcl-2* in apoptosis

There is more evidence to document the role of the *bcl-2* gene than any other known to be involved in apoptosis. *Bcl-2* is a proto-oncogene, i.e. a gene whose excessive

activity will provide a cell with a selective advantage. *Bcl-2* achieves this effect by inhibiting the apoptotic pathway (Garcia et al., 1992; Vaux et al., 1992). Likewise decreased Bcl-2 protein levels render cells more prone to apoptotic death (Miyashita and Reed, 1993). Bcl-2 represents a novel oncoprotein which appears to enhance cell lifespan but therefore may in consequence predispose these cells to carcinogenesis.

Bcl-2 protein has been detected in most tissues where apoptotic turnover and remodelling are important, and in these tissues expression is restricted to cell populations with a naturally lengthy lifespan (Hockenbery et al., 1991). Within cells Bcl-2 protein may have more than one form (Tsujimoto and Croce, 1986) with different cellular locations (Monaghan et al., 1992; Jacobson e al., 1993) and different survival capacities (Tanaka et al., 1993).

The role of Bcl-2 appears to have been conserved throughout evolution. The molecular mechanisms controlling cell death by Bcl-2 in humans and by ced-9 in *Caenorhabditis elegans* may well be the same (Vaux et al., 1992). Although mechanism of Bcl-2 action is still unclear some genes which can regulate Bcl-2 action have recently been identified. A *bcl-2* related gene, *bax*, appears to act in opposition to Bcl-2 by formation of heterodimers *in vivo* (Oltvai et al., 1993). This suggests the ratio of *bax* to *bcl-2* expression could be crucial in determining the fate of a cell.

Boise et al. (1993) have identified a *bcl-2* related gene, *bcl-x*, that is thought to exhibit death repression activity through two gene products, Bcl-xs and Bcl-xl. Although precise details of the mechanism involved are unclear it is known that the Bcl-xl protein displays remarkable amino acid and structural homology to Bcl-2. Immunohistochemical analysis using polyclonal antisera specific for detection of mouse and human Bcl-xl and Bcl-xs detected Bcl-x immunoreactivity in a wide variety of cell

types (Krajewski et al., 1994). This study revealed a more widespread pattern of bcl-x expression than northern analysis had previously shown (Boise et al., 1993). The tissue patterns of Bcl-x expression were often different from those reported for Bcl-2 suggesting that perhaps Bcl-2 and Bcl-x regulate cell life and death at different stages of cell differentiation (Krajewski et al., 1994). Bcl-x immunostaining was reported in both mouse and human mammary cuboidal epithelial cells lining the alveoli and ducts and in myoepithelial cells, occasional fibroblasts in surrounding stromal tissue were also reported to stain positively (Krajewski et al., 1994).

Another form of *bcl-x* mRNA, known as *bcl-xβ*, which is expressed in embryonal and postnatal tissues has also been identified (Gonzalez-Garcia et al., 1994). Further Bcl-2 interacting proteins recently discovered include Bag-1, a protein which although shares no significant homology with Bcl-2 or other Bcl-2 family proteins does exhibit anti cell death activity (Takayama et al., 1995), and Bad, a protein with limited sequence homology to Bcl-2 that selectively dimerises with Bcl-xl to reverse the death repressor activity of Bcl-xl (Yang et al., 1995).

Bcl-2 can modify the action of other proto-oncogenes such as *c fos* and *c myc*, which are implicated in both cell proliferation and in cell death (Bissonnette et al., 1992). The presence of Bcl-2 will inhibit apoptotic functions of these oncogenes leaving mitotic function unaffected (Fanidi et al., 1992). Thus *bcl-2* dictates the outcome of the expression of other oncogenes in a cell. Indeed Bcl-2 can block p53 induced apoptosis (Wang et al., 1993; Chiou et al., 1994).

1.4.2 Regulatory role of *p53*, tumour suppressor oncogene in apoptosis

A tumour suppressor gene is defined as one which acts to inhibit growth and

proliferation, such that loss of its function contributes to tumour progression. Tumour suppression can also be achieved if genes are able to induce apoptotic death in severely damaged cells that could otherwise proliferate in a disregulated manner. One gene with both of these abilities is *p53*.

P53 exerts its anti-proliferative effect by causing growth arrest in the G1 phase of the cell cycle, resulting in a G1/S phase arrest (Diller et al.,1990; Michalovitz et al., 1990). During this arrest in the cell cycle any damage to DNA can be repaired (Kastan et al., 1991), but in severe cases of DNA damage *p53* initiates apoptosis, and so prevents subsequent tumorigenesis.

P53 is not essential for life, since *p53* null mice develop normally, but they are just more susceptible to tumourigenesis (Jacks et al., 1994). In mouse mammary gland, hyperplastic alveolar nodules (HANS) are the most common neoplastic lesion (Medina, 1988) and mutation of the *p53* gene or altered expression of p53 protein was frequently observed in HAN outgrowths (Jerry et al., 1993). Mutations of the *p53* gene are one of the most commonly detected abnormalities in human cancer.

P53 is recognised as an important regulator of apoptosis (reviewed in Vogelstein and Kizler, 1992). In its mutated form *p53* is more stable and has a longer half life than the wild type form and in this mutated state *p53* is no longer competent to arrest the cell cycle. As a consequence cells containing potentially destructive DNA sequences are not removed, and damaged cells proliferate.

To date, two proteins have been shown to bind p53; these are GADD45 (Kastan et al., 1992) and mdm-2, a protein with oncogenic potential (Kern et al., 1991; Levine et al., 1991; Wu et al., 1993). GADD45 transcription is increased in response to DNA damage, therefore levels of GADD45 increase as p53 levels also increase

(Fornace et al., 1989). An increase in mdm-2 occurs in a p53 dependent manner (Barak et al., 1993) and the binding between the two gene products is not easily reversible (Barak and Oren, 1992). This suggests p53 and mdm-2 function in a feedback loop, to regulate the duration of cell cycle arrest following DNA damage (Chen et al., 1994).

P53 is also involved in modifying the action of other oncogenes. It has been shown to suppress expression of *bcl-2* in the murine cell leukaemic cell line M1, while simultaneously stimulating an increase in *bax* expression (Mitashita et al., 1994). This identifies *bcl-2* and *bax* as *p53* regulated genes. Indeed mice deficient in *p53* exhibit alterations in Bcl-2 and Bax protein levels in several tissues (Mitashita et al., 1994). Recently Miyashita and Reed (1995) suggested that *bax* is a *p53* primary response gene, presumably involved in a *p53* regulated pathway for induction of apoptosis.

1.5 Biological importance of apoptosis

There is now sufficient evidence to suggest that apoptosis provides a biologically meaningful function by regulating cell number and serves as a process to eliminate cells whose survival might be harmful to the animal.

1.5.1 Apoptosis during fetal life

Many cells die during normal development in both vertebrates (Glucksmann, 1950) and invertebrates (Truman, 1984). These deaths appear to be an integral part of morphogenesis and metamorphosis (reviewed in Ellis et al., 1991). The most widely reported programmed cell death during embryonic and foetal development is the death

of cells during development of the nematode worm, *Caenorhabditis elegans*. To make a worm 1091 cells must be generated and 131 of these must die at pre-ordained times (Sulston and Horvitz, 1977). Cells of many different types die in this process. In many cases the life span of a condemned cell, from genesis to death, is less than an hour. (Yuan and Horvitz, 1990).

1.5.2 Apoptosis regulates tissue mass

Apoptosis also plays a vital role in regulating adult tissue mass. The process of apoptosis is particularly prevalent in slowly-proliferating cell populations, such as liver epithelium (Kerr et al., 1972) and in adrenal cortex epithelium (Wyllie et al., 1973), where it plays an opposing role to mitosis in the regulation of tissue size. Over a period of time the rates of the two processes will balance each other, so tissue mass remains stable, i.e. there is homeostasis. Apoptosis has also been reported to occur in more rapidly proliferating cell populations, such as in intestinal crypt epithelium (Potten, 1977) and in differentiating spermatogonia (Allan et al., 1987). In these systems apoptosis will occur as cells are shed or are lost due to migration, and the rate of mitosis must then match the rate of apoptosis for tissue mass to remain stable.

1.5.3 Apoptosis regulating disease progression

Tumour development may result from disruption of the apoptotic pathway to confer a survival advantage. Apoptosis is also well recognised as a distinct pathological mechanism in tumours responding to anticancer therapies (Eastman, 1990), and so manipulation of apoptosis in tumour cells holds promise for successful cancer therapy (Williams, 1991).

In the immune system cell morphogenesis is under apoptotic control (Duke et al., 1986; Lucas et al., 1991: Stacey et al., 1985; Waring, 1990). The removal of neutrophils from inflammatory sites involves macrophage recognition and apoptosis (Savill et al., 1989, Savill, 1990), and this prevents release of granule contents so tissue injury associated with inflammation is limited. This may prove a useful point of therapy for autoimmune or chronic inflammatory disease, such as rheumatoid arthritis.

1.5.4 Apoptosis in tissue involution

Tissue involution due to physiological development or pathological change is often apoptotic in nature. Pathological conditions such as the reduction in parotid tissue after ductal obstruction (Walker et al., 1976) or acute infantile spinal muscular atrophy (Fidzianska et al., 1990) result in apoptosis of otherwise healthy tissue. Non-pathological tissue involution often represents a natural and genetically-programmed event during which healthy tissue is removed from the organism because its functioning is no longer required. Overian follicular atresia (O' Shea et al., 1978), removal of the endometrium at oestrous (Sandow et al., 1979) and death of hair follicles (Weedon and Strutten., 1981) are all examples of apoptotic cell death resulting in the removal of tissue no longer serving an essential function.

1.6 Apoptosis in mammary tissue

The removal of mammary tissue after weaning of the young is a programmed event which prevents the unnecessary channelling of energy into unwanted milk production.

Mammary epithelial cells have been reported to die by apoptosis when involution of the gland occurs (Walker et al., 1989). Mammary apoptosis is reviewed in detail in section 1.12.

1.7 The purpose of mammary development

To study the apoptotic process in the mammary gland some understanding of the development and function of the mammary gland is required. The principal function of the mammary gland is the production of milk for the nourishment of the young. The basic principle underlying the whole of mammary development, therefore, is that the mammary glands must be capable of producing milk of a quantity and composition which is appropriate to the needs of the young throughout their pre-weaning development. A relationship between the number of fetuses carried and mammary growth or milk yield have been demonstrated in a number of species (rats; Tucker, 1966; sheep; Rattray et al., 1974; goat; Hayden et al., 1979). Young mice are capable of influencing mammary development both *pre partum* and *post partum* (Knight and Peaker, 1982b). This is perhaps particularly important in communally nesting species, such as mice.

1.7.1 Domestic and laboratory species

This basic principle has been modified somewhat in dairy species, in that both the yield and composition of milk produced, and hence the gland's development, have been altered to suit man's own requirements, rather than those of the young. This has been achieved by a combination of selective breeding and adjustment of the animal's

physiology, normal habitat, environment and diet. The majority of laboratory species have also been selectively bred and this may have altered their natural pattern of mammary development. Particularly appropriate examples are strains of mice which have been bred for tumour susceptibility.

1.8 The pattern of mammary development

Unlike most other tissues, mammary gland development is not fully complete at puberty. In rodents and ruminants there are two distinct phases of gland development. Prior to pregnancy the gland is only poorly developed and consists mainly of ducts and fat, with some lobulo-alveolar secretory tissue. At this time, waves of cell proliferation and regression coincide with oestrus and luteal phases, respectively, of the oestrus cycle of pubertal animals (Sinha and Tucker, 1969). Cyclical fluctuations in cell death also occur throughout the menstrual cycle in human breast tissue (Ferguson and Anderson, 1981). After first conception mammary development consists of periods of proliferation, secretion and involution to coincide with pregnancy, birth, growth and weaning of successive litters.

The rate of milk synthesis by the mammary gland changes throughout lactation in response to many factors, including nutrition of the animal, circulating hormones and also local intramammary factors. It is the number and activity of secretory cells that determine the capacity for milk synthesis. Previous studies in the mouse have shown that changes in mammary cell number both in pregnancy and during established lactation play a significant role in attaining peak milk yield and its subsequent decline at weaning (Knight and Peaker, 1982a).

1.8.1 The lactation cycle

Each lactation cycle involves three overlapping stages. The first of these is the growth phase, termed mammogenesis when there is extensive proliferation of secretory cells during gestation and during early lactation. In rats and mice there is a 2-3 fold increase in the size of the secretory cell population during gestation (Munford, 1963).

The second phase is lactogenesis, the development of the capability for synthesis of milk components during late gestation (lactogenesis stage I) followed by the onset of copious secretion at around parturition (lactogenesis stage II; Fleet et al., 1975). This process of milk secretion is referred to as galactopoiesis.

The third phase, involution, is designed to remove excess mammary mass from the mother. It commences in late lactation, when the young are no longer entirely dependent upon their mother for nutrition. There is a gradual loss of cells, the rate of which is accelerated dramatically once the young are weaned and lactation has ceased (Ota, 1964). The extent of tissue removal during involution may vary from species to species (Hurley et al., 1989), but in rodents the mammary gland reverts to a small, relatively quiescent structure similar to that found at the start of pregnancy (Walker et al., 1989).

1.9. Phases of mammary development

1.9.1 Mammary development during gestation

In the later stages of pregnancy mammary weight increases progressively in mice, rats and goats. Methods to determine the size of a mammary population assume DNA content per cell is constant and that changes only occur in one cell type, i.e. the

epithelial cell population and not fat or connective tissue cell populations (Paape and Sinha, 1971). During pregnancy the alveolar epithelium begins to bud out from the branching duct system and replaces the fatty stroma. In mice, total mammary DNA increases exponentially in this period, doubling time being 6 days in mice at late gestation (Knight and Peaker, 1982a). Cell proliferation, as measured by tritiated thymidine incorporation is highest at mid-pregnancy in mice and falls slightly at the end of gestation (Knight and Peaker 1982a). Similar observations were made in rats (Munford, 1963) where 60% of mammary development occurs during pregnancy (Griffith and Turner, 1961).

In mice, ß casein appears at approximately day 8 of pregnancy, whey acidic protein at approximately day 14, and α lactalbumin may only be synthesized immediately after parturition (Goodman and Schanbacher, 1991) although very low levels of α-lactalbumin have been detected in the blood of pregnant animals, prior to tight junction formation between mammary epithelial cells (Mao and Bremel, 1990; 1991). In rats and pigs, α-lactalbumin is synthesized prior to parturition (Wilde and Kuhn, 1979; Buttle, 1993).

An increase in the size of the mammary population in ruminants occurs during gestation, but this growth rate is slower than that of rodents, reflecting the difference in both the size of the glands and the length of gestation (Anderson et al., 1982). At the end of gestation, cellular differentiation commences (Wilde et al., 1986) and epithelial cell size increases (Foster, 1977) possibly as a result of accumulation of secretion.

1.9.2 Mammary development during lactation

1.9.3 Mice

During early lactation mammary weight increases progressively in rodents (Tucker & Reece, 1963; Traurig, 1967). In mice this increase in mammary mass is apparent until day 10 of lactation and coincides with an increased milk yield over the same period. Peak milk yield is attained on day 10-15 of lactation (Knight and Peaker, 1982a, Shipman et al., 1987). Proliferation studies using tritiated thymidine demonstrate DNA synthesis during the first five days of lactation (Knight & Peaker, 1982a) suggesting *post partum* growth is a mechanism of adjusting the milk yield to the milk requirement of the suckling young (Knight and Peaker, 1982a). Indeed, mammary growth can also be stimulated by increasing the number of young in a litter, but this effect is only apparent during the first few days of lactation (Knight at al., 1986). This may be important for those species, such as mice, which have a communal existence and cross-suckle (Matthews, 1952). Until day 5, cell hypertrophy is modest compared to the extent of cell proliferation indicating that the increase in milk yield may be ascribed to increased cell proliferation rather than increased synthetic capacity per cell (Shipman et al., 1986; 1987). After 5 days any increase in milk yield is most likely to be attributable to hypertrophy of secretory cells (Shipman et al., 1986: 1987).

1.9.4 Rats

In rats between days 2 and 7 of lactation milk yield increases by 80%, cell number by 55% (Knight et al., 1984). At this time the elevation in milk yield is attributable to cell number and cell differentiation. During the second week of the lactation cycle milk yield increases a further 20% and cell number increases by 20% (Knight et al

1984). Therefore milk yield increases to a peak at day 14 as does the size of the mammary population along with the DNA:RNA ratio (Knight et al., 1984). In rats beyond day 16 of lactation milk yield falls.

1.9.5 Goats

In goats over the first three weeks of lactation both milk yield and DNA content of the gland (a measure of the size of a mammary population) increase (Anderson et al., 1981; Knight & Peaker, 1984), and mammary cell proliferation does not cease at parturition in goats but continues into the early stages of the lactation cycle (Knight and Peaker, 1984). Between weeks 3 and 8 although the size of the mammary population remained constant, yield steadily increased over this period to reach a peak at around week 8 of lactation (Blatchford and Peaker 1982; Knight and Peaker, 1984). This increase can be attributed to an increase in cell differentiation (Wilde et al., 1986). In goats a steady decline in milk yield occurred between weeks 8 and 23. The size of the mammary cell population falls by some 30% and mammary udder volume falls over this period in line with decreases in milk yield observed in these animals (Knight and Peaker, 1984).

1.9.6 Mammary cell loss during tissue involution

Natural involution is a gradual process which commences during declining lactation as the young start to eat solid food. When suckling ceases involution is accelerated. Involution of the lactating human breast following weaning is accompanied by a rapid decrease in weight (Ota, 1964: Carlsson et al., 1973; Helminen et al., 1968), in the proportion of the organ occupied by glandular parenchyma (Williams, 1942; Wellings

and Deome, 1963; Richards and Benson, 1971), and in total DNA content (Griffith and Turner, 1962; Tucker and Reece, 1963; Ota, 1964; Carlsson et al., 1973). During mammary involution there is also a reduction in cell size (Helminen and Ericsson, 1968; Brandes et al., 1969; Richards and Benson, 1971; Wellings and Deome, 1963).

1.10 Control of mammary development

1.10.1 Control of mammary development during gestation

During pregnancy, mammary growth is stimulated by a combination of steroids and polypeptide hormones. The steroids include a mixture of oestrogens, which are responsible for increasing the number of proliferating cells while decreasing the time required for cell division in mice (Bresciani, 1971) and in rats (Grahame and Beralanffy, 1972).

Polypeptide hormones also regulate mammary growth during gestation. These protein hormones include prolactin, growth hormone and placental lactogen, the contribution each plays *pre-partum* varies according to the species. In many species, placental lactogen is secreted by the placenta and is a major contributor to lactogenic activity during gestation in ruminants (Kelly et al., 1976; Forsyth, 1986; Collier et al., 1995), while in others placental lactogen is secreted in such minute amounts as to make no significant difference to mammary development (Cowie et al., 1980). Placental lactogen levels are higher in animals carrying a large number of foetuses, resulting in a degree of mammary growth appropriate to the needs of the young (Knight and Peaker, 1982b; Flint et al., 1995). Mammogenesis is induced by placental lactogen, however the mechanism involved is presently unclear, and probably does not involve

prolactin, IGF-1 or somatotrophin receptors (Collier et al., 1995).

In women placental lactogens are present in substantial amounts in the second half of pregnancy (Franks et al., 1977, Nielson et al. 1979; Prichard et al., 1985). In mice and rats coitus results in twice daily prolactin surges and in rising prolactin levels. In ruminants prolactin levels remain low until a few days before parturition as in goats placental lactogen is largely responsible for the lactogenic activity and levels of this hormone to rise prior to mid-pregnancy and plateau between day 110 and parturition at day 150 (Knight et al., 1982a). Rising levels of prolactin at parturition offsets the loss of plactental lactogen from the system (Cowie et al., 1980).

1.10.2 Control of mammary development in lactation

Control of mammary development involves a complex hierarchy of interacting mechanisms including regulation by systemic endocrine control, regulation exerted by any foetuses *in utero* and by the degree of suckling *post partum* . Finally, the influence of environment, diet and the metabolic status of the mother are also crucial to the overall development pattern during lactation.

Lactation is maintained by hormonal influences which differ between species. In rodents it was thought that primarily prolactin and not growth hormone regulated both lactogenesis and galactopoiesis. However, it has been shown that growth hormone increases in importance as lactation progresses (Flint et al., 1992), particually when circulating prolactin concentrations are low (Madon et al., 1986; Barber et al., 1992; Flint et al., 1992). Recently a local galactopoitic effect of growth hormone has been demonstrated in rats (Flint and Gardiner, 1994) which cannot be mimicked by IGF-1, or IGF-2, IGF analogues or complexes of IGF's and binding protein (Flint et al.,

1994), showing that growth hormone is acting via an IGF independent pathway.

In ruminants it is growth hormone and not prolactin which provides the main galactopoietic stimulus (Karg et al., 1972; Hart and Flux, 1973). Growth hormone can maintain milk secretion in the absence of prolactin in hypophysectomized lactating goats (Cowie et al., 1964), and stimulates milk production in intact goats (Knight et al., 1990) and cows (Bauman et al., 1985).

Despite extensive study of the mechanisms of hormone action in the mammary gland, the mode of growth hormone and prolactin action is unclear. A number of experiments *in vitro* using mammary tissue explants and isolated cells have shown that the expression of the genes for the major milk proteins, the caseins, are regulated by prolactin at the level of transcription and mRNA stability (Guyette et al., 1979). However, casein mRNA is detected in the rat during pregnancy at a time when serum prolactin is relatively low (Rosen and Barker, 1976). The factor that confers prolactin stimulation to milk protein gene transcription has recently been identified as mammary growth factor (MGF) or STAT5 (Liu et al., 1995). This is a transcription factor that becomes activated by a tyrosine specific protein kinase, Jak 2. Tyrosine phosphorylation converts STAT5 from a latent factor into one with DNA binding and transcriptional potential (Groner and Gouilleux, 1995). The regulation of MGF / STAT5 *in vitro* and *in vivo* indicate that it is a central component of the lactogenic hormone signaling pathway (Ashkenas et al., 1995; Kazansky et al., 1995; Streuli et al., 1995b).

Growth hormone can produce a local mammogenic response when infused into individual glands of pregnant heifers (Collier et al., 1993) and the mitogenic effects of growth hormone have been confirmed in goats using *in vivo* bromodeoxyuridine

Growth hormone receptor mRNA has been identified and characterised in many organs including the mammary glands of many species (Jammes *et al.*, 1991; Tiong and Hermington, 1991). The expression of growth hormone receptor mRNA was subject to gestational, developmental and tissue specific regulation (Ilkbahar *et al.*, 1995).

labelling to quantify cell proliferation (Knight et al., 1994). However the growth hormone receptor has not yet been demonstrated on mammary epithelium (Akers, 1985). It is likely that the mammogenic effect of growth hormone is mediated by IGF-1, a recognised mammary mitogen (Winder and Forsyth, 1986). The IGF-1 receptor is thought to be present in mammary stromal tissue (Hauser et al., 1990), and growth factors including IGF-1 administered locally can elicit a growth response in mammary tissue (Collier et al., 1993).

1.10.3 Co-ordination of mammary involution

Mammary epithelial cells are removed from the gland at the end of a lactation cycle. Dying mammary epithelial cells have been reported to show nuclear changes, which have since been shown to be characteristic of cells undergoing apoptosis (Wellings and Deome, 1963; Martinez-Hernandez et al., 1976). Epithelial cells in involuting tissue were lysed after being shed into the lumen (Wellings and DeOme, 1963; Richards and Benson, 1971). Myoepithelial cells have variously been reported to show little change (Helminen and Ericcson, 1968; Radnor, 1972) in some studies, and to undergo cytolysis in a manner similar to epithelial cells in other studies (Wellings and DeOme, 1963). Rapid shrinkage of the capillary bed is known to accompany regression of the mammary parenchyma (Williams, 1942), but how this is accomplished is unknown.

Loss of the suckling stimulus results in decreased production of lactogenic hormones, milk stasis within the gland (Wilde et al., 1995a) and the expression of metalloproteinases (Strange et al., 1992). During involution the alveolar structure disintegrates and the basement membrane is removed by ECM-degrading metalloproteinases. A delicate balance between the expression of these proteinases and

their inhibitors controls the breakdown of gland structure (Talhouk et al.,. 1991, 1992). Initially, the proteinases are inhibited by a temporary increase in inhibitors, which prevents the immediate breakdown of the basement membrane. After a few days, tissue inhibitor of metalloproteinases (TIMP) expression decreases while metalloproteinase expression remains, this favours degradation of the basement membrane (Strange et al., 1992). During the involution process milk protein synthesis is switched off and is accompanied by regression of the alveoli and a reduction in tissue mass. This remodelling process leads to a phenotype similar to that seen in the virgin animal (Walker et al.,1989).

1.11. Basement membrane control of mammary function

Many cells require interaction with basement membrane for full differentiation and function. In these systems gene expression and controlled differentiation are reliant upon cell-extracellular matrix (ECM) interactions. In the hepatocyte albumin gene expression is due to control by laminin (Bissell et al., 1987, Liu et al., 1991) and in the skin, terminal keratinocyte differentiation occurs when basement membrane contact is lost in suprabasal layers (Watt et al., 1988; Kubler et al., 1991).

Mammary cells cultured on plastic dishes fail to produce milk proteins even under hormonal conditions optimal for lactation. In contrast, when these cells are cultured on an extracellular matrix extracted from Engelbreth Holm Swarm tumour (EHS) containing laminin, collagen I and IV, fibronectin and vitronectin, these cells recreate their alveolar structure and synthetic phenotype (Aggeler et al., 1991; Barcellos-Hoff et al., 1989; Li et al., 1987). Mammary epithelial cells plated onto

EHS secrete caseins and whey acidic protein unidirectionally into the sealed central lumina (Barcellos-Hoff et al., 1989; Chen and Bissell, 1989). Transferrin, on the other hand, as *in vivo,* is secreted bidirectionally and in culture is recovered in the lumina and culture medium (Barcellos-Hoff et al., 1989; Chen and Bissell 1989).

1.12. Apoptosis during mammary involution

1.12.1 Initial identification of mammary apoptosis

At the start of this study, in October 1992, mammary apoptosis had been shown to occur during involution of rodent mammary tissue at the end of lactation (Walker et al., 1989) and after litter removal at peak lactation (Walker et al., 1989; Strange et al., 1992). The sequence of events during apoptosis were studied by light and electron microscopy (Walker et al., 1989), by detection of DNA laddering (Strange et al., 1992) and by changes in mammary gene expression (Strange et al., 1992). From these studies apoptosis was shown to occur in epithelial, myoepithelial and endothelial mammary cell populations (Walker et al., 1989). After weaning, macrophages in the tissue were found to increase in size as they became filled with lipid vacuoles and lipofuscin pigment granules due to phagocytosis of apoptotic bodies (Walker et al., 1989). DNA laddering, visualised by the rather insensitive ethidium bromide method detected laddering one day post-weaning when litter removal occurred at peak lactation and laddering increased to a peak at 4 days before decreasing to barely detectable levels at six days post weaning (Strange et al., 1992). Control of apoptosis by prolactin demonstrated the hormonal dependence of apoptosis in mammary tissue (Sheffield and Kotolski, 1992).

The timings of these changes coincided with changes in the pattern of gene expression in mammary tissue. Strange et al., (1992) extracted RNA from involuting glands and analysed for the expression of genes associated with

1) epithelial differentiation - *ß casein, whey acidic protein*

2) tissue remodelling - *TIMP, stromelysin*

3) stress and cell death - *heat shock protein 70, tissue transglutaminase*

4) regulation of cell proliferation and diferentiation - *c myc, p53, TGF ß*

Expression of *ß casein* and *whey acidic protein* were dramatically reduced during involution induced by litter removal at peak lactation (Strange et al., 1992). Changes in the expression of proteases were detected within the initial two days post weaning, whereas protease inhibitor levels remained high until three days post weaning. At 6 days post weaning protease levels fell, whilst levels of inhibitors rose (Strange et al., 1992). Genes involved in the stress responses were induced by weaning as where genes associated with regulation of cell growth and differentiation (*p53* and *TGF ß* at 1 day post weaning; *c myc* at 4 days post weaning; Strange et al., 1992).

1.12.2 Characterisation of mammary gene expression associated with apoptosis

Study of gene expression during mammary involution has identified changes in known and uncharacterised genes in the process of mammary apoptosis. For example, weaning induced a decrease in the expression of *poly (ADP-ribose) polymerase* during mammary apoptosis (Guenette et al., 1994) and the immediate early genes *c fos* and *c jun* are also elevated during the early stages of mammary apoptosis (Marti et al., 1994). Differential display techniques identified a range of genes with altered expression patterns during the early stages of mammary apoptosis. One of these is the

gas 1 gene which is upregulated during mammary involution (Bielke et al.,1995) and appears to exert its effects through *p53* by causing growth arrest (Schneider et al., 1988, Del Sal et al., 1992). It may be induced by loss of cell adhesion to extracellular anchors.

Further studies using sophisticated cell culture techniques have demonstrated the importance of integrin mediated control of mammary development (Streuli et al., 1993) and a role for *bcl-2* and *bax* have been demonstrated during mammary involution (Edwards et al., 1995). Recent cell culture experiments have demonstrated the importance of interleukin (IL) 1ß converting enzyme (ICE) during mammary apoptosis (Boudreau et al., 1995). This enzyme is a protease which cleaves pro IL-1ß to generate mature IL-1ß. ICE is homologous to proteins that have been implicated in apoptosis in other systems, including *ced 3* (Yuan et al., 1993).

1.13 Aims of this project

Research to date has shown that mammary cell loss during tissue involution is by apoptosis, and that this process is at least in part, under endocrine control. Characteristic changes in gene expression during apoptosis have served to identify at least some of the events likely to control this process during involution. There has, however been very little knowledge of the process of apoptosis in mammary tissue and very little published information in this area. Involution and apoptosis had almost without exception been induced by litter removal (Walker at al., 1989; Strange et al., 1992) or natural weaning (Walker at al., 1989) in rodents; whether similar events occur under more natural concurrent pregnancy conditions is unclear. A comparision of the

extent and timing of events during the apoptotic process has not been performed. Likewise, virtually nothing is known about the regulation of mammary apoptosis. The extent of DNA laddering in mammary tissue has been determined by several investigators using insensitive ethidium bromide staining techniques, and it has therefore been perceived that apoptosis has either only been restricted to tissue involution or that internucleosomal nicking has not been an important feature of mammary apoptosis.

To address these areas in this project I have compared the time-course and extent of apoptosis in rodents after induction by litter removal at both peak and declining lactation and during natural weaning. Events were also compared to those observed during concurrent pregnancy, to determine the necessity of apoptosis in this situation and to build a more comprehensive picture of the role of apoptosis in rodent mammary tissue.

Besides determining the importance of apoptosis throughout lactation and involution, some of the extracellular and intracellular factors that regulate apoptosis were also investigated. It is likely that factors responsible for regulation of lactation are also involved in control of apoptosis.

Endocrine regulation of lactation has been well established, and manipulation of mammary apoptosis by prolactin had been previously reported (Sheffield and Kotolski, 1992). Investigation into the effectiveness of prolactin and growth hormone on altering the rate of mammary apoptosis aimed to provide information about the hormonal regulation of apoptosis and also to show the relative importance of these two hormones during rodent lactation.

Lactation is also regulated by local control mechanisms. For example, frequent

milking in goats has been demonstated to alter milk yield and mammary cell number (Henderson et al., 1983, 1985; Knight and Wilde, 1987; Wilde et al., 1989, 1991). Milk stasis had been shown to control milk secretion, and furthermore a constituent of milk has been isolated and shown to regulate milk secretion by autocrine feedback inhibition (Wilde et al., 1995a). Therefore the possibility of regulation of apoptosis by local mechanisms resulting from milk stasis were investigated.

Although the role of apoptosis in rodents has been studied at least to some extent, the importance of apoptosis during involution of mammary tissue in other animals was unknown. Despite extensive study of the lactation cycle of ruminants, with a view to extending the lactation cycle of dairy animals or improving milk yield during lactation, the role of apoptosis during lactation and involution has not been determined. It has been suggested that the extent of cell carryover between lactation cycles in ruminants may be minimal compared to the situation in rodents, implying that cell death may not be an important feature of ruminant involution (Hurley et al.,1989). Knowledge of the extent of apoptosis during involution of ruminant mammary glands will bring greater understanding of the mechanisms operating within mammary tissue during involution of the gland. This may suggest strategies for controlling mammary cell number and potentially for extending lactation in dairy animals or altering cell carryover between lactation cycles to improve subsequent milk production.

Throughout lactation cell number changes in a controlled manner, and in the goat a substantial decrease in cell number has been noted during the declining phase of lactation (Knight and Peaker, 1982 a,b). This raises the possibility that apoptosis may be responsible for cell removal during this declining phase of lactation. Indeed apoptosis may even prove to be an important regulatory mechanism at all stages of

lactation. A more successful adjustment of mammary cell number during lactation may be possible once the interplay between proliferation and death mechanisms has been determined. This would allow lactation to be more finely tuned than is presently possible.

The dynamic balance between mammary proliferation and cell death the occurence and extent of mammary apoptosis has been studied in this project in rodents and ruminants *in vivo*. However to understand more about the regulation of mammary apoptosis it is most likely that the use of mammary cell culture systems will prove advantageous. The employment of cell culture models allows the use of a more precisely defined and less complex system than *in vivo*. Unfortunately normal mammary cells are both stucturally and functionally compromised in many of the mammary cell culture systems that are in regular use (Ip and Darcy, 1996). In particular in monolayer culture studies neither differentiation nor alveolar structure is maintained and synthesis of whey acidic protein, an important milk constituent, is not achieved by monolayer culture of epithelial cells despite hormone conditions optimal for milk secretion (Chen and Bissell, 1989). However one cell culture system has been developed whereby epithelial cells are cultured on a layer of reconstituted basement membrane (Barcellos-Hoff et al., 1989; Chen and Bissell, 1989; Streuli and Bisssell, 1990; Aggeler et al., 1991). In this environment epithelial cell are polarised and secrete milk in a similar manner to mammary epithelial cells *in vivo* (Li et al., 1987; Schmidhauser et al., 1990; Seely and Aggeler, 1991, Streuli et al., 1991). This system has become the one of choice when investigating mechanisms of milk production and secretion. It is hoped that mammary epithelial cells grown on reconstituted basement membrane will prove suitable for the study of apoptosis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Radiochemical were from ICN Flow, Irvine, UK. Cell culture media were purchased from either Gibco BRL Ltd., Paisley, UK or Northumbria Biologicals Ltd., Cramlington, UK. Molecular biology reagents were purchased from Promega, Southampton, UK or Boehringer Corp. Ltd., Lewes, UK. General laboratory chemicals were supplied by Sigma Chemical Co, Poole, UK or BDH, Poole UK unless stated otherwise.

Mouse cDNA probe for stromelysin-1 was a gift from Dr. L.M. Matrisian, Department of Cell Biology, Vanderbilt University, Nashville, Tennessee, USA. The mouse cDNA probe for tissue inhibitor of metalloproteinase 2 (TIMP-2) was a gift from Dr. M. Cockett, Celltech Ltd., Slough, UK. cDNA probes for *p53* were provided by Dr. T. Crook, Ludwig Institute for Cancer Research, London, UK and Professor M. Oren, The Weizmann Institute of Science, Rehovot, Israel. *bcl-2* cDNA was a gift from Dr. Y. Tsujimoto, Osaka University Medical School, Osaka, Japan.

Water used in any molecular biological procedure was molecular biology grade from Sigma Chemical Company, Poole, UK.

2.1.2 Animals

Mice derived from Tuck's No. 1 strain (A Tuck and Sons, Battlebridge, UK) and female Wistar rats were provided with food and water *ad libitum* and housed as described previously (Knight et al. 1986). All animals were used in their first lactation unless stated otherwise. In mice and rats, litter size was adjusted to 10 pups on day 1 of lactation (the day immediately following nocturnal parturition).

British Saanen goats from the Institute herd were routinely milked twice daily at approximately 08.00 and 16.00 h. They were fed 1.5-1.8 kg concentrates daily (16.5% crude protein, 12.5 MJ/kg; Goat mix No. 1, Edinburgh School of Agriculture, Edinburgh, UK), receiving half of the ration at each milking, with hay and water available *ad libitum*.

2.2 Experimental procedures

2.2.1. Mouse experiments

Mouse litters were removed from the dam into a separate cage on day 10 or day 16 of lactation and mammary tissue was collected from the dam 0, 1, 2, 4 or 6 days thereafter.

Mammary tissue was also obtained from two groups of lactating dams on alternate days, between days 16 and 26 of lactation. The first group of dams was suckling their first litter, the second group of dams was mated at *post partum* oestrus and so the animals were lactating their first litter while pregnant with their second litter.

Mouse mammary teats were selectively sealed with tissue adhesive (Vetseal;

Braun Melsungen AG, Melsungen, Germany) at day 10 of lactation. Pups were removed from the dam for 1-2 h while the sealant was allowed to dry, and the size of the litter was reduced on return to the dam to maintain a one pup to teat ratio. These pups were allowed to suckle *ad libitum* for 1 or 2 days, before the removal of mammary tissue.

2.2.2 Rat experiments

Experiments in three groups of rats commenced on day 12 of lactation. In the first group litters were removed for 24 and 48 h in the first group. The effects of hormone withdrawal were investigated in the second group. Prolactin was removed by daily intraperitoneal injection of bromocriptine whilst anti growth hormone serum was administered by daily subcutaneous injection to neutralise the effect of growth hormone. Mammary tissue was taken after 24 and 48 h of individual or combined anti hormone treatment. In the third group of rats the effects of hormone replacement were determined in animals previously treated with bromocriptine and anti growth hormone serum. Prolactin replacement or growth hormone replacement for 24 h was followed by *post mortem* tissue collection. Tissue from treated rats was compared with lactating rat mammary tissue collected on day 12, 13 or 14 of lactation.
Previous studies have demonstrated the efficacy of these treatments (Madon *et al.*, 1986).

2.2.3 Goat experiments

Two experiments were performed in goats. In one experiment twelve goats were milked twice daily throughout lactation prior to a unilateral milking treatment in which one gland remained unmilked for up to 3 weeks whilst the other continued to be milked twice daily. After 2 days, 1, 2 or 3 weeks of treatment, *post mortem* tissue samples

were taken from each mammary gland.

In a second experiment three groups of goats were allowed to suckle their young immediately after parturition, to establish milk secretion. These goats were then switched to one of three unilateral milking regimes for four weeks:-

1) the right gland milked three times a day; the left gland milked once daily

2) the right gland suckled by the kids *ad libitum*; the left gland milked once daily

3) the right gland drained continuously after insertion of a catheter into the teat canal; the left gland milked once daily.

Biopsy tissue was taken from each mammary gland prior to unilateral treatment in the first group of goats and from each mammary gland at the end of unilateral treatment in all three groups of goats.

2.3 Cell culture

2.3.1 Cell culture media

Mammary cells from mid pregnant mice were cultured as described previously (Blatchford et al., 1994). Details of the culture media are given in table 2.1. All media, pH 7.4, were sterilised through a 0.2 μm pore filter (Gelman Sciences, Southampton, UK) prior to use. Hormones were prepared for addition to the culture media as follows:

Insulin (0.1mg/ml) was prepared by dissolving 1 mg of insulin (from bovine pancreas, 24.4 I.U./mg) in 1 ml of water containing 10 μl of 0.34 M NaOH. This stock was made up to 10 ml with water and aliquots were filter sterilised and stored at -20°C.

Cortisol (0.1 mg/ml) was prepared by dissolving 1 mg of cortisol (hydrocortisone-21-

acetate) in 1 ml of ethanol. The solution was diluted to 10 ml in water and aliquots stored at -20°C.

Prolactin (from sheep pituitary, 32 I.U./mg) was prepared by dissolving 1mg of oPrl in 250 μl of 10 mM Hepes (pH 8.0), diluted with water to 10 ml and aliquots were filter sterilised and stored at -20°C.

Triiodothyronine (T_3) 0.65 mg was dissolved in 250 μl of 0.34M NaOH and diluted to 10 ml with water. Aliquots (10 μl) were filter sterilised and stored at -20°C, and diluted to 10 ml with water as required.

2.3.2 Preparation of mammary cells

Mice killed by cervical dislocation were rinsed in 70% (v/v) ethanol, prior to the collection of mammary tissue (approximately 10 g). Excess milk was removed by washing in 20 ml of Hanks buffer (pH 7.4) and the tissue was finely chopped using curved scissors. Digestion was preformed in 40 ml of digestion medium (1 x Hanks Basal Salt Solution, 1 x Minimum Essential Amino Acids, 5 mM glucose, 2 mM glutamine, 4% bovine serum albumin. 5 μg / ml insulin, 1 μg/ ml cortisol, 167 U / ml Collagenase class 111), (Worthington Biologicals Ltd, U.K.) using an orbital incubator (Gallenkamp, Loughborough, UK) at 37°C and 120 rpm with agitation by pipette every 15 min. After approximately 100 min, when the cells were predominantly in clumps of 10-20 cells, the digest mixture was filtered through a 150 μm pore nylon mesh (Henry Simon Ltd., Stockport, UK) held in a 45 mm filter support (Nalgene, Rochester, New York, USA). The cells were harvested by gentle centrifugation (80 *g*, 4 min, room temperature), resuspended in 20 ml of wash medium (1 x Hanks Basal Salt solution, 1 x Minimum Essential Amino Acids, 5 mM glucose, 2 mM glutamine, 10 mM Hepes, 5 μg / ml insulin, 1

μg / ml cortisol, 200 μg / ml trypsin inhibitor, 40 U / ml DNase) and reharvested. This wash step was repeated three times. The final cell pellet was filtered through a 50 μm pore nylon mesh and resuspended in 5 ml of culture medium (1 x Medium 199, 1 x Hams F12, 21 mM Hepes, 0.4 % sodium bicarbonate, 5 μg / ml insulin, 1 μg /ml cortisol, 1 μg / ml prolactin, 650 pg / ml Tri-iodothyronine, 1 mM glucose, 10 % foetal calf serum). Insulin levels were reduced to 0.5 μg / ml in experiments determining IGFBP levels to ensure insulin did not react with IGF receptors. Cell viability was assessed by trypan blue exclusion.

Cells were dispensed into 6 well plastic tissue culture plates (Costar, Cambridge, Mass., USA) and incubated at 37°C under 5% CO_2 (v/v) in a saturated water atmosphere. After a 1 h equilibration period, the cells were harvested by centrifugation (80 *g*, 4 min) and resuspended in 2 ml of fresh incubation medium. Cells were layered onto a freshly prepared continuous Percoll gradient, (1.01 - 1.06 g/ml) and separated by centrifugation according to density (80 *g*, 20 min). Layers containing mainly clumps of cells were pooled and cell yield was estimated from an aliquot of the cell suspension by comparision of the packed cell pellet volume after centrifugation (11,000 *g*, 30 s) with a predetermined standard.

Extracellular matrix, prepared in-house, was carefully thawed below 4°C, and dispensed into plastic tissue culture plates (2.5 ml matrix / 35mm diameter well). After spreading evenly at 4°C, the matrix was polymerised (15 min, 37°C). 1 x 10^6 epithelial cells in 3 ml culture medium were then dispensed into each culture well. Culture medium, without foetal calf serum, was replaced daily.

Cells were harvested by replacing culture medium with 0.4 ml Dispase (Universal Biologicals Ltd., London, UK). After incubation (20 min, 37°C) cells

released from the matrix were collected and centrifuged (1,000 *g*, 2 min), washed with 0.5ml fresh culture medium, and again centrifuged (1,000 *g*, 2 min). The cell pellet was stored immediately in liquid nitrogen for subsequent analysis. Other cells were fixed for histological study by addition of freshly prepared 4% (w/v) paraformaldehyde in 0.1M phosphate buffer to each well (3 x 30 min washes). Fixed cells were washed in 0.1M phosphate buffer pH 7.4, containing 2% sucrose and 0.02% calcium chloride, 3 x 10 min), and then stored in 1M sucrose at 4 °C until required for embedding.

Extracellular matrix consists of the extracellular extract from Engelbreth - Holm - Swarm mouse tumour, as described on page 79.

2.4 DNA analysis

2.4.1 Extraction of DNA

Frozen mammary tissue was ground to a powder under liquid nitrogen, and incubated for 3 h at 56°C in 10 volumes (w/v) of 10 mM Tris-HCl pH 8.0 containing 1 mM EDTA, 0.5% (w/v) SDS and 100 μg/ml proteinase K. The digest was extracted with phenol pH 8.0 (3 x 1 volume) and chloroform:isoamylalcohol (24:1 (v/v), 1 x 1 volume), incubated with 5 μg / ml RNase A for 18 h at room temperature and genomic DNA was precipitated with ethanol. DNA was resuspended in 10 mM Tris-HCl pH 8.0 containing 1 mM EDTA, and yield was assayed by $A_{260/280}$ ratio.

Alternatively, DNA was prepared using a Puregene DNA Isolation Kit, (Flowgen, Sittingbourne, UK) according to the manufacturers instructions, except that frozen mammary tissue was homogenised in liquid nitrogen prior to mixing with the cell lysis solution and samples were treated with RNase for 3 h rather than for 15 - 60 min. When DNA was extracted from frozen cells after culture experiments the cell pellets were resuspended in 600 μl of cell lysis solution and samples were treated with

In the initial stages of the project, laddering of DNA on agarose gels was detected by ethidium bromide staining. The same method had been used in all previous studies of mammary apoptosis (refs). This method proved both insensitive and of low reproducibility. Similar conclusions have been drawn when this method was used with other tissues (Oberhammer et al., 1992). In many samples, visualisation of DNA laddering by ethidium bromide binding required overloading of DNA to an extent which exceeded the linear range for ethidium bromide fluorescence. Indeed, in several instances where cell death was by other criteria apoptotic, DNA electrophoresis and ethidium bromide staining did not detect laddering (Collins et al., 1992; Lockshin et al., 1991; Tomei, 1991; Zakeri et al., 1993).

The shortcomings of ethidium bromide staining could not be overcome by varying the conditions for gel staining e.g. by increasing DNA loading or ethidium bromide concentration. Thus, although laddering (as a proportion of total DNA) could be in theory be quantitated for individual samples by densitometry of gels photographed under UV illumination, providing that sample loading and staining conditions were optimal, this was not possible in practice. Visualisation of oligonucleosomal ladders required DNA overloading to the extent that intact DNA was not quantifiable. Moreover, comparison of sequential samples necessitated constant DNA loading with the result that, for example, early-involution laddering was undetectable or late involution laddering was excessive. Finally, it was found that the method was unreliable. The same samples gave markedly differing results when analysed on several occasions. From this we concluded that ethidium bromide staining was impracticable for the studies we wished to undertake in mammary tissue.

An alternative to ethidium bromide staining for detection of DNA laddering was developed from a method used successfully with ovarian tissue (Rösl, 1992). This method depends on nick end-labelling of fragmented DNA with [^{32}P]-deoxynucleotide. Nick end-labelling obviated the difficulties of sensitivity and reproducibility associated with ethidium bromide labelling. For example, DNA laddering was observed reproducibly at peak lactation when ethidium bromide staining failed to detect any laddering. Similarly, repeated assay of standard samples under the same conditions produced similar results, which were measured by [^{32}P] Cerenkov counting of incorporated radiolabel prior to gel electrophoresis and autoradiography, for example a sample of DNA from a mid lactation mouse after two days of litter removal gave Cerenkov counts of 10,520 ±260 cpm / μg DNA. This approach was used to quantitate apoptosis induced after litter removal in mice and during endocrine manipulation in rats. Both studies have been accepted for publication (Quarrie et al., 1995; Travers et al., 1996).

In adopting nick end labelling as a means of quantifying cell death by apoptosis, some limitations to the method must be recognised. The cumulative intensity of signal for the "rungs" in the ladder is a measure of oligonucleosomal DNA cleavage, and cannot be directly compared with ethidium bromide staining which occurs by intercalation with DNA, and whose intensity is a function of both cleavage and length of fragment generated. Thus, the intensity of the lowest rungs of the ladder tended to be greatest, this was not the case for ethidium bromide staining. In addition, oligonucleosomal fragments will subsequently be cleaved by non-specific endonuclease; and for quantitation to be accurate, considerable care must be taken to avoid degradation of DNA during analysis.

RNase for 3 h rather than 15 - 60 min, otherwise the protocol was followed according to the manufacturer's instructions.

2.4.2 DNA electrophoresis

DNA samples (10 μg) were subjected to electrophoresis in 1.8% (w/v) agarose gels prepared in 40 mM Tris-HCl (pH 8.2) containing 20 mM sodium acetate, 1 mM EDTA and 5 μg/ml ethidium bromide, and photographed under UV illumination.

2.4.3 End labelling of DNA

DNA (10 μg) was diluted with an equal volume of 100 mM Tris-HCl pH 8.0 containing 10 mM $MgCl_2$ in a siliconised microcentrifuge tube, and incubated with 30 nCi of $\alpha[^{32}P]$-dCTP and 2.5 U of DNA polymerase 1 (Klenow fragment) for 10 min at room temperature. The reaction was stopped by addition of EDTA (final concentration, 10 mM), and the reaction volume was adjusted to 100 μl with water. DNA was recovered by ethanol precipitation in the presence of 20 μg glycogen, and resuspended overnight in 10 mM Tris-HCl pH 8.0 containing 1 mM EDTA. Incorporation of $\alpha[^{32}P]$-dCTP radioactivity into samples was determined using a liquid scintillation analyzer 1600 Tr (Canberra Packard, Pangbourne, UK). DNA samples (1 μg) were subjected to agarose gel electrophoresis as described above. Equal loading of DNA was confirmed by UV illumination before the gel was fixed in 7% (w/v) trichloroacetic acid for 20 min. The gel was dried, sealed in plastic and subjected to autoradiography at -80 C. **This method is a modification of a method described by Rosl (1992).**

Refer to text explaining limitations of this method inserted on page 37

2.4.4 DNA assay

DNA was measured by a fluorometric procedure (Labarca and Paigen, 1980). The

DNA standard (calf thymus) was prepared in water at 1 mg/ml at 4°C, and diluted to 10 μg/ml with assay buffer (0.1 M NaH_2PO_4, pH 7.4 containing 2 M NaCl) before use. Fluorescent reagent was prepared by dissolving bisbenzimide (Fluka, Glossop, UK) in water at 1 mg/ml and diluted to 3 μg/ml with assay buffer before use.

Tissue samples were sonicated (15 s at setting 30; Kontes micro-ultrasonic cell disruptor, (Burkard Scientific, Middlesex, UK) in assay buffer and an appropriate volume was removed for assay. The DNA standard was used in the range 0 - 4 μg and all assay volumes were made up to 1ml with assay buffer before addition of 0.5 ml of fluorescent reagent. After 15-20 min at room temperature, fluorescence was measured in a DNA TKO 100 minifluorimeter (Hoeffer Instruments, San Francisco, USA).

2.5 Histology

2.5.1 Tissue collection

Female mice and rats were killed by cervical dislocation. Mammary tissue was removed immediately and portions were snap-frozen in liquid nitrogen or fixed in freshly prepared 4% (w/v) paraformaldehyde in 0.1M phosphate buffer pH 7.4 for 1 h. Fixed tissue was then transferred into 0.1M phosphate buffer pH 7.4 for subsequent histological analysis.

2.5.2 Histological analysis

Fixed tissues were dehydrated through graded alcohols and chloroform, and paraffin embedded. Sections (4 μm) were cut on a Reichert-Jung Autocut 2040 microtome and attatched to poly-L-lysine subbed slides. Paraffin wax was removed from the sections

by incubating at 56°C for 30 min before immersing in xylene or Histoclear (Agar Scientific, Stansted, UK). Thereafter, sections were taken through graded alcohols to water so that staining could commence.

Fixed cells from culture experiments were scraped from the tissue culture plate with a spatula and transferred to a 1.5 ml microcentrifuge tube with a wide bore pipette. The cells were pelleted (80 *g*, 5 min, room temp) and the supernatant was removed. The pellet was gently resuspended in OCT freezing compound over 5 min to form a capsule of cells and OCT. This capsule was lowered into liquid nitrogen, the microcentrifuge tube coating was removed and the capsule was embedded onto the cryostat chuck at -80°C. Frozen sections (5 μm) were cut and air-dried for 24 h before staining.

For examination of tissue morphology, sections were stained with Meyer's haematoxylin (20 min, room temperature), followed by "blueing up" in warm tap water (5 min) and staining in eosin (1 min, room temperature). Sections were then rinsed in water, dehydrated through graded alcohols and xylene, before mounting in DPX (Agar Scientific, Stansted, UK)

For inspection of chromatin, sections were stained in diamidinophenylindole (DAPI; 1 ng/ml) for 5 min, rinsed in water and mounted in Vectashield (Vector laboratories, Peterborough, UK).

Milk protein production was investigated by staining with fluorescently-labelled polyclonal antisera raised in-house against total casein, ß casein, whey acidic protein and transferrin. After 2 h of antibody staining, sections were rinsed in water and mounted in Vectasheild (Vector laboratories, Peterborough, UK).

Distribution of laminin in tissue sections, was determined with an anti-laminin

mouse antibody (TCS biologicals, Botolph Claydon, U.K.), followed by a rabbit anti mouse biotinylated second antibody and finally visualised using an avidin biotin complex (ABC) Horse radish peroxidase was linked to the avidin-biotin in the ABC complex kit (Dako, High Wycombe, UK) at room temperature for 30 min and followed by 0.6 mg/ml diaminobenzidine for 2-5 minutes. Sections were then rinsed in water, dehydrated through graded alcohols and xylene, before mounting in DPX (Agar Scientific, Stansted, UK).

2.5.3 *In situ* end labelling of DNA

Sections were digested with 0.5% (w/v) pepsin at ambient temperature for 15 min, and end labelled using 1mM biotinylated deoxyuridine triphosphate and 1 U/ml DNA polymerase I (Klenow) at 37°C for 30 min as described by Ansari et al., (1993). In some cases pepsin treatment was replaced by 50 μg/ml proteinase K at 37°C for 15 min. Endogenous peroxidases were inactivated by incubating sections with 3% (v/v) hydrogen peroxide at room temperature for 15 min. End labelling was then revealed by sequential incubation with the ABC kit (Dako, High Wycombe, UK) at room temperature for 30 min, and 0.6 mg/ml diaminobenzidine. Sections were counterstained with Meyer's haematoxylin, dehydrated and then mounted in DPX.

2.6 RNA studies

2.6.1 RNA extraction

RNA was prepared by the method of Chirgwin et al., (1979). Frozen mammary tissue (0.01 - 0.05 g tissue) was ground to a powder under liquid nitrogen, and vortexed in 3.5 ml of RNase inhibiting buffer (0.05 M Tris-HCl pH 7.5, 0.01 M EDTA pH 7.5 containing 4.22 M guanidium thiocyanate, 5% Sarkosyl and 1% 2-mercaptoethanol). DNA was sheared

by passing the homogenate through 21G and then 23G needles, and caesium chloride (1.4 g) was added. This was then layered above a 1.5 ml caesium chloride cushion (5.7 M caesium chloride pH 7.5, 0.1 M EDTA) and centrifuged (149,000 *g*, 20°C, 17 h). The RNA pellet was resuspended in water in a sterile tube and impurities were removed by chloroform:butanol (4:1, 2 x 1 volume). RNA was precipitated with ethanol, resuspended in water and assayed by $A_{260/280}$ ratio.

Alternatively, RNA was prepared using the Ultraspec II RNA isolation system (AMS Biotechnology Ltd., Witney, Oxon, UK). Frozen tissue samples were ground in liquid nitrogen before adding to the Ultraspec RNA reagent. Frozen cell pellets were mixed with the Ultraspec RNA reagent. The manufacturer's protocol was then followed exactly. RNA was resuspended in water and assayed by $A_{260/280}$ ratio.

2.6.2 RNA electrophoresis

RNA samples (5 μg or 40 μg) were loaded onto a 1.2% agarose gel prepared in 20 mM MOPS pH 8.0 containing 1 mM EDTA pH 8.0, 8 mM sodium acetate, 1.85% (w/v) formaldehyde and 0.5 μg/ml ethidium bromide. Samples were subjected to electrophoresis at 60 V for approximately 2 h. The gel was photographed under UV illumination to confirm equal loading of samples, and then was washed in 10 x SSC [1 x SSC is 0.15 M sodium chloride pH 7.0, 0.015 M sodium citrate pH 7.0). RNA was transferred overnight to Biotrans nylon membrane (ICN Flow, Irvine, UK) and was firmly attached to the membrane by UV crosslinking (Spectrolinker XL-1000, Spectronics Corporation, New York, USA). The membrane was then wrapped in plastic and stored at 4°C.

2.6.3 Preparation of cDNA probes

Plasmids containing cDNA inserts (1-10 ng) encoding mRNAs of interest were transformed into *E.coli* JM109 cells by standard procedures (Hanahan et al., 1985). Tranformants were plated on agar plates containing 25 μg/ml ampicillin. After overnight incubation at 37°C, colonies growing on the agar were selected and grown in 50 ml of LB broth (10 g bacto-tryptone, 5 g bacto yeast extract, 10 g NaCl per litre of water, pH 7.0). Plasmid DNA was then extracted from the cells by alkaline lysis (10 mM sodium hydroxide followed by 3 M potassium acetate) following the method of Sambrook et al., (1989). DNA was treated with RNase (20 μg/ml, 37°C, 1 h), and 2 μl was resolved on a 1% (w/v) agarose gel in 0.05 M Tris pH 7.5 containing 0.05M boric acid, 1 mM EDTA and 5 μg/ml ethidium bromide for 2 h at 70 V, to ensure intact, high quality plasmid had been isolated.

Insert DNA was digested from vector DNA with restriction enzymes according to the restriction sites present on the vector and insert (Table 2.1).

cDNA	Vector	Insert	Restriction enzymes to isolate insert
Stromelysin	pBR322	1.5-1.6 kb	EcoR1
TIMP-2	pSP73	0.8 kb	Hind 111 and BGL 11
p53	pGem	1.3 kb	EcoR1

Table 2.1 Preparation of cDNA probes. Information essential for ensuring the correct cDNA probe is isolated for northern blotting.

After overnight incubation, at 37°C, of DNA with a restriction enzyme, the DNA was ethanol precipitated and resuspended in water. DNA was resolved on a 1% (w/v) low melting point agarose gel (Gibco BRL, Paisley, UK) in 40 mM Tris-acetate (pH 8.2), 1 mM EDTA and 0.5 μg/ml ethidium bromide, and photographed under UV illumination.

The insert was excised from the gel, placed in a microcentrifuge tube and 3 ml of water was added for every gram of gel. The mixture was boiled for 10 min and then stored at -20°C.

On the day of use the cDNA probe was boiled for 3 min and 12.5 ng was incubated in a labelling reaction, containing 2.5 μl of OLB, 0.5 μl of 10 mg/ml BSA, 12.5 μCi of $\alpha[^{32}P]$-dCTP and 2U of DNA polymerase I (Klenow), to a total reaction volume of 25 μl, for 2.5 h at room temperature (Feinberg and Vogelstein, 1984).

Radiolabelled DNA was separated from unincorporated label on a 2 ml G50 Sephadex column. The $\alpha[^{32}P]$-dCTP activity of DNA was counted on a liquid scintillation analyzer 1600TR (Canberra Packard, Pangbourne, UK), and the DNA was precipitated, then resuspended in 50 μl of water and stored at -20°C for up to one week.

2.6.4 Northern blotting with cDNA probes

Filters were pre-hybridised (42°C for 4 h, 2 h on each side) in hybridisation buffer (50% (v/v) deionised formamide, 5 x SSC, 200 μg/ml salmon sperm DNA, 0.1% (w/v) lauryl sulphate, 5 x Denhardts solution and 0.05% (v/v) pyrophosphate tetrasodium) using a hybridisation oven (Appligene, Newcastle, UK). The radiolabelled cDNA probe was boiled for 10 min and then added to the hybridisation tube in 10 ml of fresh

hybridisation buffer for 18 h.

Filters were removed from the hybridisation oven and washed on a shaking platform (3 x 100 ml; 10 min room temperature in 2 x SSC pH 7.0, containing 0.1% (w/v) lauryl sulphate), followed by washing in a pre-heated waterbath (1 x 100 ml of 0.2 x SSC pH 7.0, containing 0.1% (w/v) lauryl sulphate), at increasing 10°C intervals until only hybridised probe remained on the filter. At this point the filter was sealed in plastic and subjected to autoradiography.

2.6.5 Design of oligonucleotides

Cross-species 30-mer oligonucleotide probes for *bcl-2* and *bax* were designed from accession sequences for *bcl-2* and *bax* mouse, rat and human gene sequences using computer programs.

Oligonucleotides were identified and designed using the Blast Gateway to USA computer programme.

Bcl-2 oligonucleotide sequence corresponded to 434 - 463 bp (Tsujimoto and Croce, 1986) 3' CCTCCTAACACCGGAAGAAACTCAAGCCAC 5'

Bax oligonucleotide sequence corresponded to 174 - 203 bp (Oltvai et al., 1994)

3' CTACGCAGGTGGTTCTTCGACTCGTTCTGT 5'

Bax GenBank accession numbers were:- human _bax_ - L22473.gb-pr, L22474.gb-pr, L22475.gb-pr, murine _bax_ - L22472.gb-ro, rat _bax_ - L22471.gb-ro, human _bcl-2_- L15048.gb-pr, murine _bcl-2_ - L 15047.gb-ro, rat _bcl-2_ - L15049.gb-ro.

2.6.6 Northern blotting with oligonucleotide probes

Oligonucleotide 30-mer sequences (10 μg) were labelled with 1 μCi of [^{32}P]-dATP, using 0.008 U of T4 polynucleotide kinase (37°C, 30 min). Unincorporated label was separated from labelled DNA using a 2 ml column. The [^{32}P]-dATP activity of

labelled DNA was counted on a Liquid Scintillation Analyzer 1600 TR (Canberra Packard, Pangbourne, UK) and DNA was ethanol precipitated, resuspended in 50 μl of water and stored at -20°C for up to one week.

Filters were prehybridised for 4 h (2 h each side) in 10 ml of hybridisation buffer (6 x SSC, 5 x Denhardts, 0.1% (w/v) lauryl sulphate, 0.05% (v/v) pyrophosphate tetrasodium and 200 μg/ml salmon sperm DNA) in the hybridisation oven. Radiolabelled oligonucleotide probes were added to the hybridisation tube in 10 ml of fresh hybridisation buffer for 18 h.

Filters were removed from the hybridisation oven and washed on a shaking platform (3 x 100 ml washes in 6 x SSC pH 7.0 containing 0.1% (w/v) lauryl sulphate for 10 min, at room temperature), followed by washing in a pre-heated waterbath (1 x 100 ml of 6 x SSC pH 7.0 containing 0.1% (w/v) lauryl sulphate for 30 min) at Tm - 12 °C. At this point the filter was sealed in plastic and subjected to autoradiography.

2.6.7 Densitometry

RNA from at least three animals at each time-point was electrophoresed under denaturing conditions. This RNA was visualised by ethidium bromide staining and photographed, the relative amounts of 28S RNA loaded onto each lane on the gel were determined using a video densitometer (Biorad, UK). The densitometer scanned each lane at least three times and an average value was obtained. Autoradiographs, depicting expression patterns of various cDNA probes were also scanned by the densitometer, each lane on the autoradiographs was scanned at least three times in the area of interest for each probe. The whole track area was also scanned on autoradiographs containing

bax or *TIMP-2* expression data, since expression of more than one transcript was revealed by northern analysis. The average value of expression obtained for each lane as normalised according to RNA loading. The yield of RNA /g tissue, which had been noted during extraction of RNA, varied considerably between lactating tissue, where RNA yield was approximately 10.9mg /g tissue, and involuting tissue, where RNA yield could be as low as 2.0 mg /g tissue. Therefore normalised RNA expression values were expressed per unit RNA to take into account differences in tissue RNA levels. This method elimimates the requirement for an internal control or housekeeping gene.

Day to day control of RNA blots was achieved by including the same two control samples of RNA on each RNA gel. The controls comprised one sample of RNA from lactating mouse mammary gland and one sample of RNA from involuting mouse mammary gland. Expression levels from one blot to the next can then be normalised according to the level of expression from the two control samples, after expression was corrected for variations in RNA loadings.

CHAPTER THREE

APOPTOSIS IN MOUSE MAMMARY TISSUE

3.1 Introduction

It is now well established that cell removal during mammary involution is by programmed cell death i.e. apoptosis (Walker et al., 1989; Strange et al., 1992; Tenniswood et al., 1992; Atwood et al., 1995). The progression of apoptosis during involution was followed by light and electron microscopy (Walker et al 1989), while changes in an extensive range of genes expressed during the involution process have been compared with the progression of apoptosis in mouse mammary tissue, to discover some of the important genetic changes that regulate mouse mammary apoptosis (Strange et al., 1992, Tenniswood et al., 1992). Throughout these studies DNA laddering was visualised by ethidium bromide staining of electrophoresed DNA (Walker et al., 1989, Strange et al., 1992).

In all of these studies mammary apoptosis has been studied primarily in mouse mammary tissue after litter removal in peak lactation, a somewhat unnatural *in vivo* situation. To date, apoptosis induced at other timepoints in the lactation cycle, by mechanisms other than litter removal, has received very little attention. This study has examined the time course and extent of apoptosis induced by litter removal in declining lactation and during natural weaning, conditions which represent more natural manipulations of the involution process. The processes of natural weaning have been

studied in both non pregnant and pregnant mice, since there is little or no time between successive lactation cycles *in vivo*. The effect of unilateral milk stasis on mammary apoptosis was also investigated to determine if apoptosis could be regulated by local control mechanisms sensitive to milk stasis, which may also, at least in part, mediate apoptosis after litter removal or during natural weaning.

3.2 Experimental protocol

The timecourse and extent of apoptosis were studied in four experimental situations.

3.2.1 Litter removal

The effects of pup removal were investigated by removing the litter at either peak lactation (day 10 of lactation) or in declining lactation (day 16 of lactation).

3.2.2 Natural weaning

The response to litter removal was compared with natural weaning. In this case the litter remained with the mother as it was weaned onto solid food, a process which began around day 16 of lactation. Tissue was collected on alternate days, for 10 days throughout the natural weaning process.

3.2.3 Natural weaning in concurrently pregnant mice

Natural weaning was studied first in non-pregnant mice. However, in other circumstances mice are often concurrently pregnant while lactating, with little intervening dry period between lactations. We wished to determine if in these

circumstances, involution and apoptosis was necessary.

3.3.4 Milk stasis

The fourth group of mice was subjected to unilateral teat sealing, such that milk stasis was induced in mammary glands on one body half. This technique provides a means of assessing the importance of local i.e. intra-mammary mechanisms in regulating mammary apoptosis.

3.3 Results

3.3.1 Litter removal at mid-lactation

Induction of apoptosis by litter removal in mid-lactation was accompanied by extensive changes in tissue morphology (Fig 3.1) such that 3 - 4 days after litter removal there was little evidence of alveolar organisation. Oligonucleosomal DNA laddering indicative of apoptosis was detected by ethidium bromide staining after 4 days of litter removal (Fig 3.2A). However, when genomic DNA was end-labelled with [^{32}P] dCTP, autoradiography revealed increasingly intense DNA laddering in tissue taken 1 or 2 days after pup removal with a further increase by four days of pup removal (Fig 3.2B). After 6 days of litter removal DNA laddering had decreased. Cerenkov counting of [^{32}P] dCTP incorporation prior to electrophoresis showed a progressive increase up to 4 days of litter removal (Table 1), consistent with the intensity of laddering. End-labelling of DNA with [^{32}P] dCTP also detected laddering on day 10 of lactation suggesting that apoptosis occurs at peak lactation (Fig 3.3). Large DNA fragments were also detected after radiolabelling DNA, this may represent intermediates generated in endonuclease cleavage prior to internucleosomal fragmentation (Brown et al., 1993;

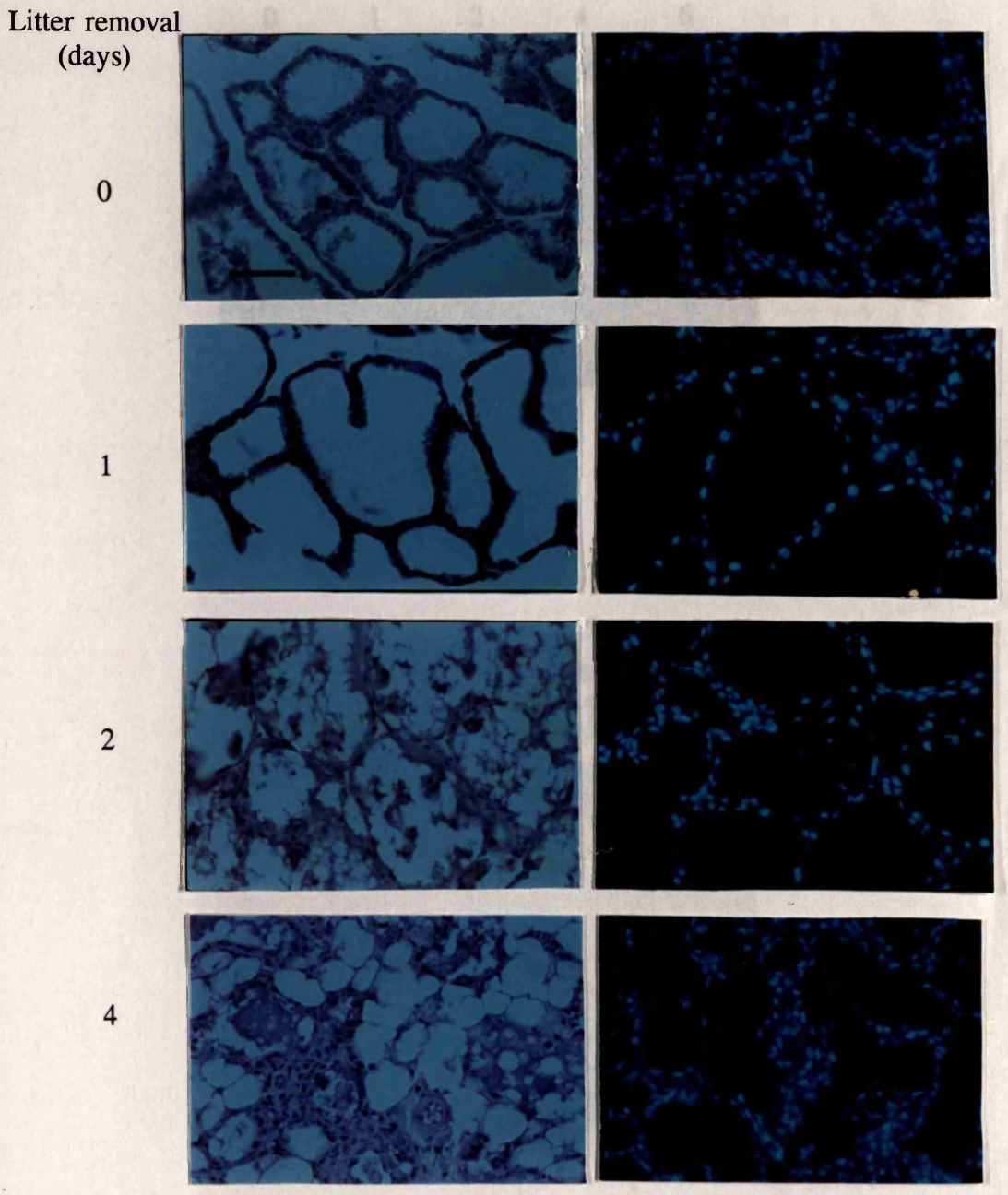


Fig. 3.1 Histology of mouse mammary tissue after litter removal in mid-lactation. Tissue was collected *post mortem* immediately and 1,2 or 4 days after iitter removal on day 10 of lactation. Tissue sections were stained with haematoxylin / eosin or DAPI. Bar = 40 μm.

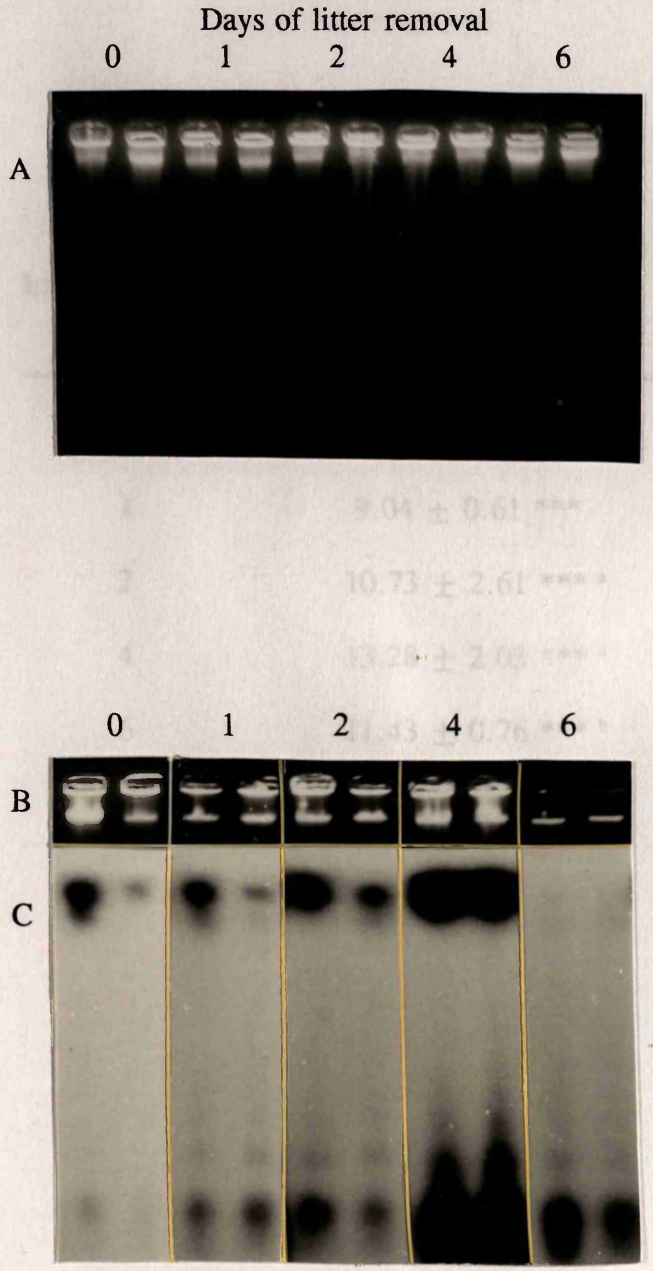


Fig. 3.2 DNA laddering in mouse mammary tissue after litter removal in mid-lactation. Tissue was collected *post mortem* immediately and 1, 2, 4 or 6 days after litter removal on day 10 of lactation. A, ethidium bromide staining of resolved DNA. B, ethidium bromide staining of unresolved DNA in sample wells confirming equal loading of samples. C, autoradiography of [^{32}P] dCTP end-labelled DNA. Results from two animals are shown at each timepoint.

Litter removal (days)	[^{32}P] incorporated (CPM/μg DNA 10^{-3})
0	4.51 ± 0.10
1	9.04 ± 0.61 ***
2	10.73 ± 2.61 *** [a]
4	13.28 ± 2.03 *** [a]
6	11.43 ± 0.76 *** [b]

Table 3.1 Quantitative analysis of DNA laddering in mouse mammary tissue. Tissue was collected *post mortem* immediately and 1,2,4 or 6 days after litter removal in mid-lactation. DNA was nick end-labelled with [^{32}P] dCTP using Klenow polymerase and counted for radioactivity (Cerenkov). Values are mean ± SEM for 4 animals, except [a], n=3. *** $P<0.001$ compared with day 10 values. [b], $P<0.05$ compared with 1 days litter removal. Statistical analysis by Student's t-test.

Fig. 3.3 DNA laddering in mouse mammary tissue at mid-lactation. Tissue obtained *post mortem* from two day-10 lactating mice (a and b). DNA was extracted from the tissue, end labelled with [^{32}P] dCTP and subjected to autoradiography.

Oberhammer et al., 1993)

3.3.2 Litter removal during declining lactation.

Although similar histological changes occur following litter removal on day 16 and day 10 of lactation, tissue involution progressed more rapidly after litter removal on day 16 of lactation than that on day 10 of lactation. After 2 days, alveolar integrity was lost and luminal spaces had filled with intact cells and apoptotic bodies (Fig 3.4). At this stage DNA content had decreased by 35% compared with day 16, and six days after litter removal, total DNA content was 36% of the pre-treatment value.

The change in DNA laddering after litter removal on day 16 was similar in time course to that induced at peak lactation. Laddering increased on the first day of litter removal, increased further on the second day, remained high on day 4 and decreased after 6 days (Fig 3.5). However laddering was consistently more intense than that observed after litter removal on day 10.

The method for end-labelling DNA with [^{32}P] dCTP was optimised using tissue from which the litter was removed in declining lactation. More [^{32}P] dCTP was incorporated into DNA with DNA polymerase 1 (Klenow) than with terminal transferase (Fig 3.6A). Precipitation of DNA end-labelled with [^{32}P] dCTP recovered more labelled DNA than using a spin column (Fig 3.6B), and glycogen produced a higher recovery of DNA after precipitation. dNTP's in the reaction mix did not influence the extent of radiolabel incorporated into DNA (Fig 3.6C).

Fragmented DNA was demonstrated in tissue sections by addition of a dUTP to the ends of fragmented DNA. From this technique fragmented DNA was end-labelled and visualised *in situ* (ISEL). Prostate and thyroid gland tissue were used

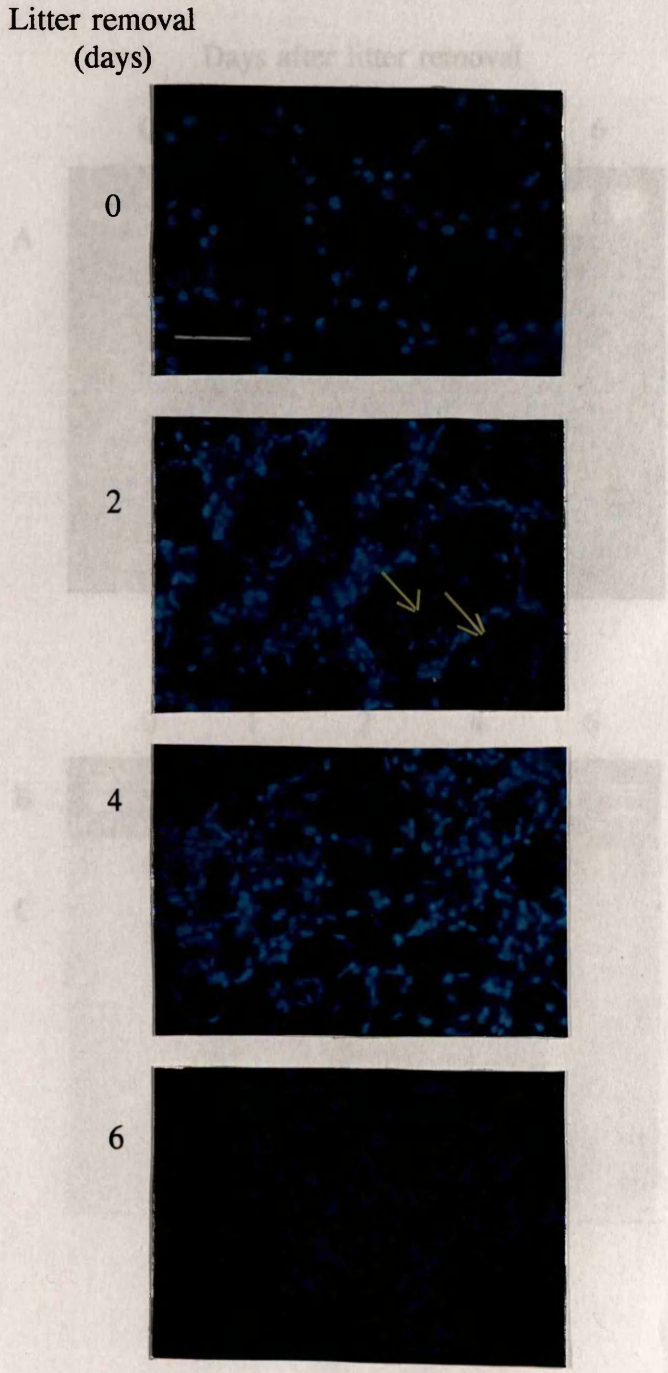


Fig. 3.4 Histology of mouse mammary tissue after litter removal in declining lactation. Tissue was collected *post mortem* immediately and 2,4 or 6 days of litter removal on day 16 of lactation. Tissue sections were stained with DAPI. Arrows, apoptotic bodies. Bar = 40 μm.

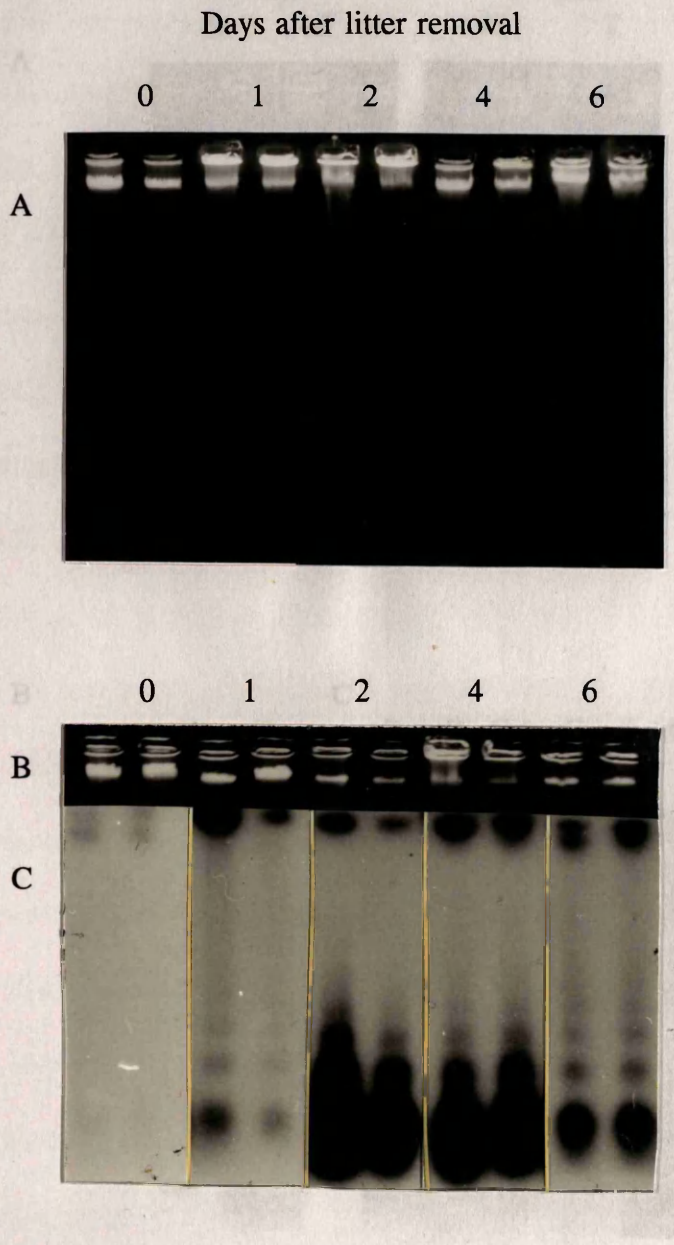


Fig. 3.5 DNA laddering in mouse mammary tissue after litter removal in declining lactation. Tissue was collected *post mortem* immediately and 1,2,4 or 6 days after litter removal on day 16 of lactation. A, ethidium bromide staining of resolved DNA. B, ethidium bromide staining of unresolved DNA in sample wells to confirm equal loading of samples. C, autoradiography of [^{32}P] dCTP end-labelled DNA. Results from two animals are shown at each timepoint.

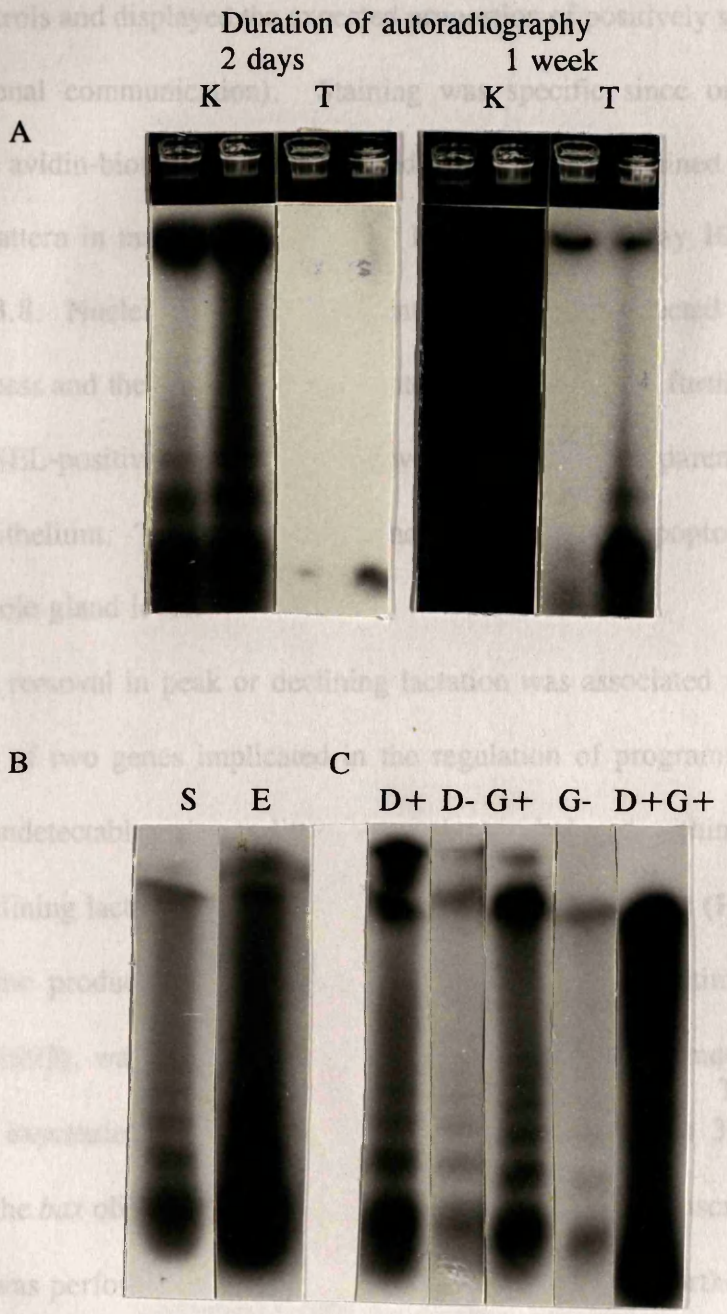


Fig. 3.6 Optimisation of nick end-labelling for visualisation of DNA ladders in mouse mammary tissue. A, Autoradiography of DNA nick end-labelled with [^{32}P] dCTP using either Klenow polymerase (K) or terminal transferase (T). B, Autoradiography of DNA nick end-labelled with [^{32}P] dCTP and Klenow polymerase. Labelled DNA was recovered by either ethanol precipitation (E) or a spin column (S). C, Autoradiography of DNA nick end-labelled with [^{32}P] dCTP and Klenow polymerase with and without dATP, dTTP and dGTP (D) included in the reaction mix, and with or without glycogen (G) included in the precipitation.

as positive controls and displayed the expected proportion of positively stained cells (B. Ansari, personal communication). Staining was specific since omission of the antibody or the avidin-biotin procedure resulted in no positively stained cells (Fig 3.7). The staining pattern in mammary tissue after litter removal on day 10 of lactation is shown in Fig 3.8. Nuclei containing fragmented DNA were detected 2 days into the involution process and the number of fragmented nuclei increased further by 4 days of involution. ISEL-positive mammary cells were distributed, apparently at random, through the epithelium. There was no evidence of coordinated apoptosis, either at an alveolar or whole gland level.

Litter removal in peak or declining lactation was associated with changes in the expression of two genes implicated in the regulation of programmed cell death. *p53* mRNA, undetectable prior to litter removal, was induced within 1 day of litter removal in declining lactation, and remained high for at least 4 days (Figs 3.9 - 3.11). *Bax* whose gene product is reported to form homodimers and stimulate apoptosis (Oltvai et al. 1993), was expressed in the early stages of litter removal in declining lactation, but expression decreased markedly after 2 days (Figs 3.9 - 3.11). It appeared that the *bax* oligonucleotide may detect several mRNA transcripts. Therefore densitometry was performed on both the whole area exposed to northern analysis and just to the area that had previously been reported to represent *bax* expression. Both analyses showed proportionately similar changes, and all the values relating to *bax* expression in this chapter are those refering to the previously reported area of *bax* expression. The detection of several mRNA species by the *bax* oligonucleotide is unlikely to represent non-specific hybridisation due to the high stringency of post hybridisation washes, but maybe the *bax* oligonucleotide is detecting other perhaps as

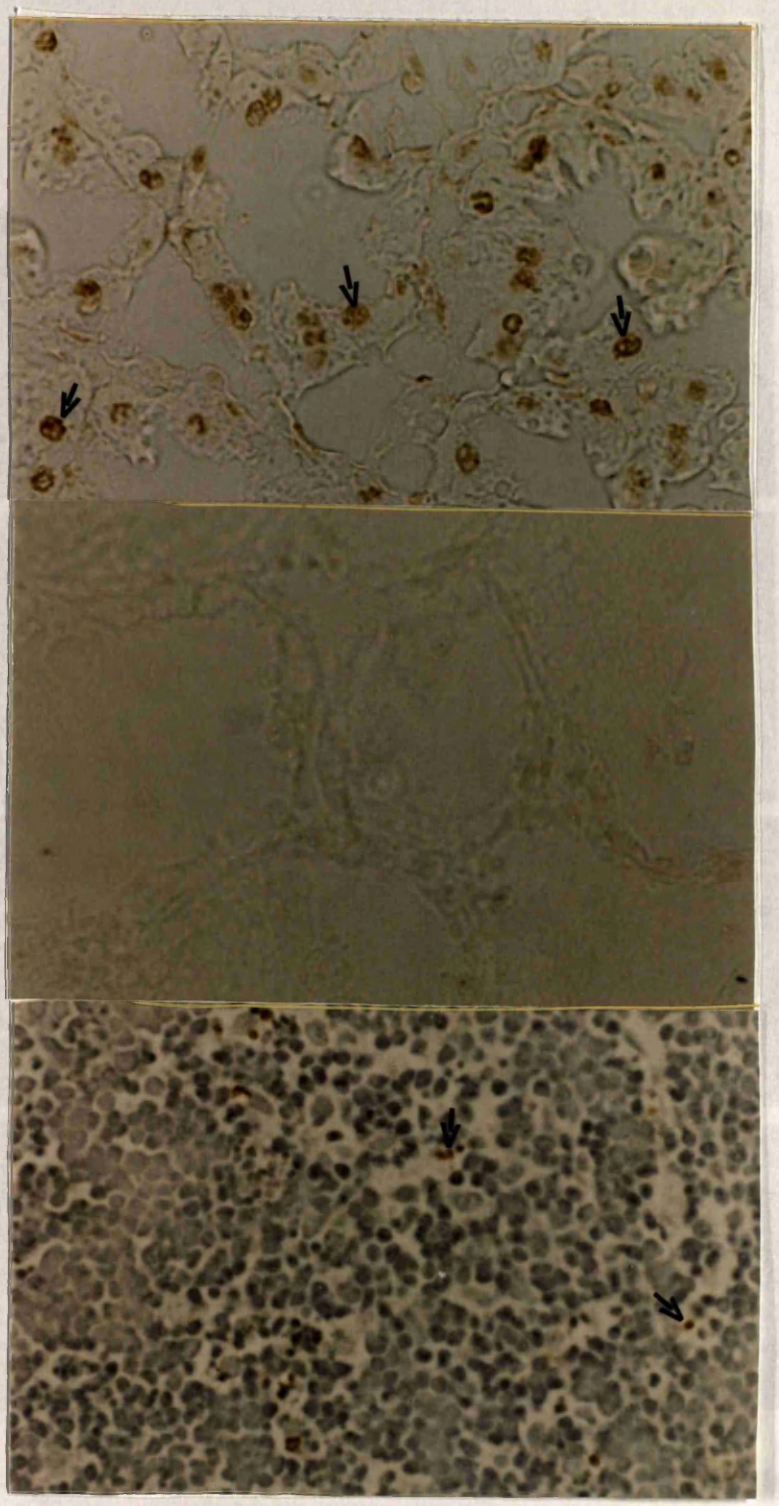
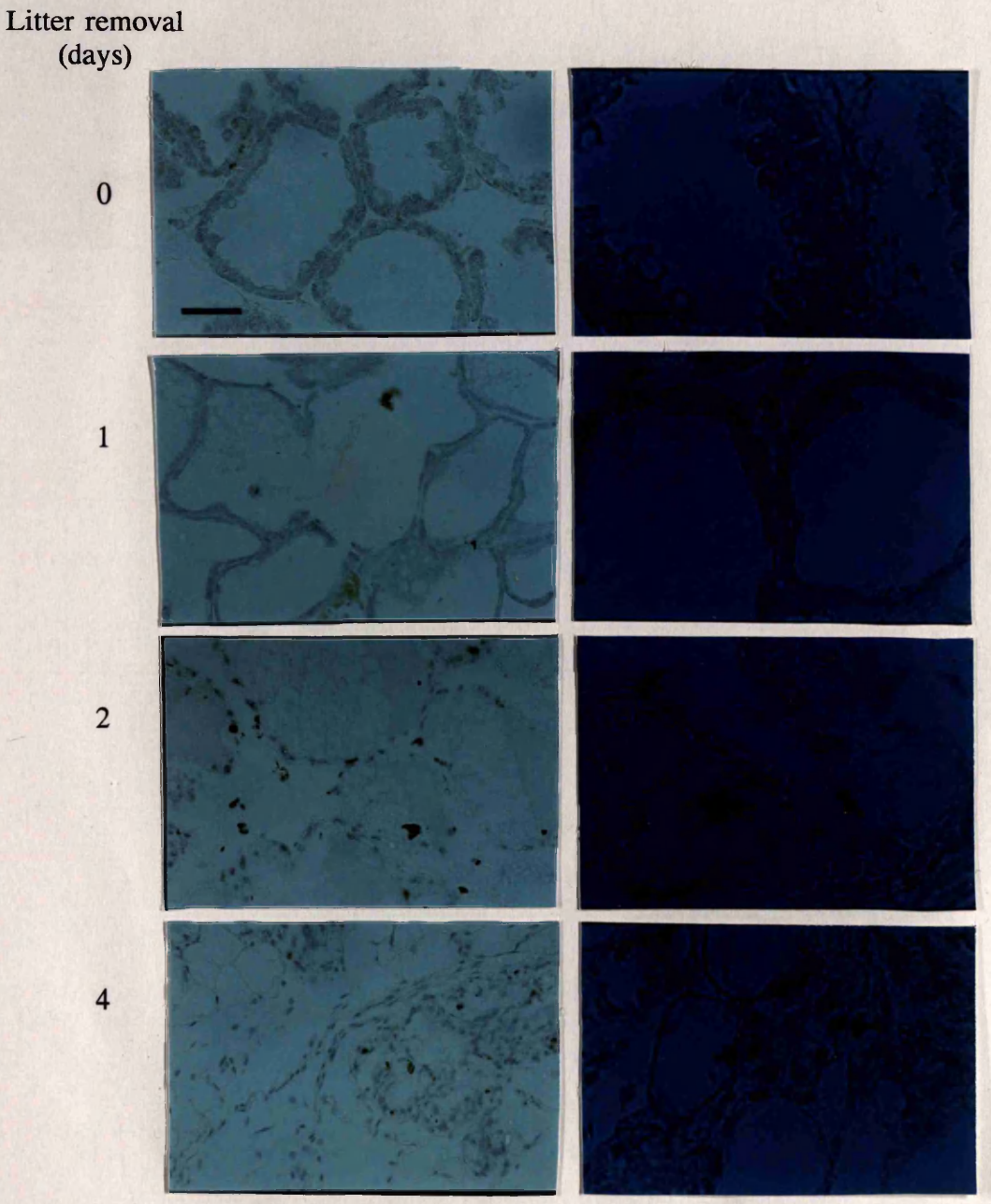


Fig. 3.7 ***In situ*** **end-labelling (ISEL) of mouse mammary tissue sections with biotinylated dUTP.** A, mouse mammary tissue section treated with DNase 1 prior to application of dUTP antibody. B, negative control, omitted avidin-biotin from the staining procedure. C, mouse thymus tissue section containing a proportion of apoptotic cells (sections courtesy of Dr. B Ansari). Arrows, cells containing fragmented DNA.



Tissue	Percentage of ISEL positive cells
lactating tissue	<0.2%
2-3 days of litter removal	1.7%
4 days of litter removal	4.5%

Fig. 3.8 ***In situ*** **end labelling (ISEL) of mouse mammary tissue sections.** Tissue was collected *post mortem* immediately and 1, 2 or 4 days after litter removal on day 10 of lactation. Arrows, cells containing fragmented DNA. A, Bar = 40 μm. B, Bar = 5 μm.

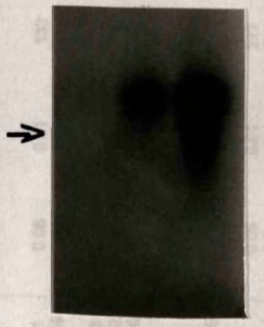
Fig. 3.9 Mouse mammary gene expression after litter removal on day 10 of lactation. Expression of *p53, bax, stromelysin* and *TIMP-2* was measured by northern analysis of total RNA. A, ethidium bromide staining of total RNA after agarose gel electrophoresis. B, northern analysis of RNA hybridised with [^{32}P] dCTP end-labelled cDNA probes. C, densitometry of hybridised RNA detected by autoradiography and corrected for RNA loading and RNA yield/g tissue. Values are the mean $\pm$ S.E.M. for 3 animals. Arrows indicate 18S ribosomal RNA.

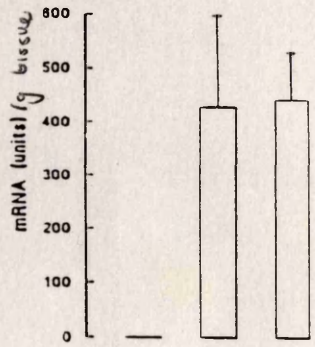
A

B

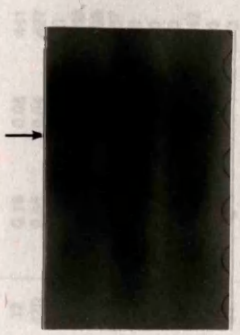
C

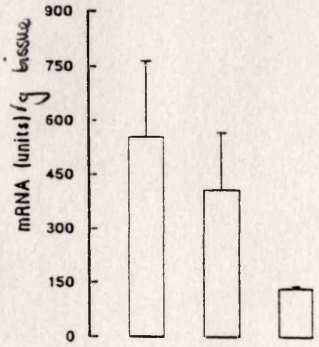
p53



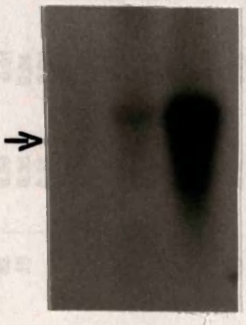


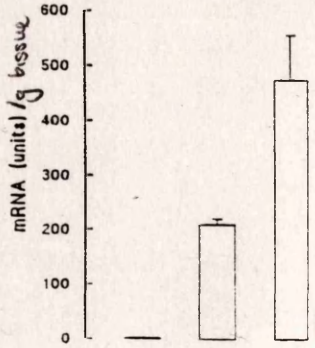
bax



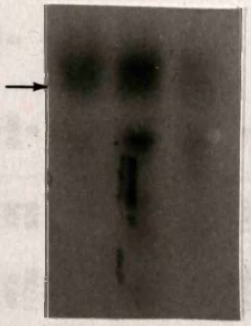


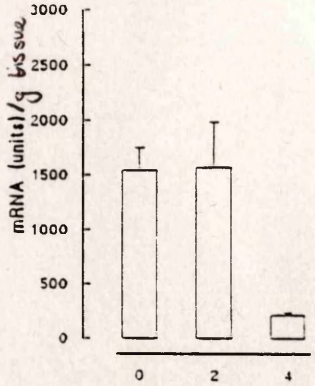
stromelysin





TIMP-2





0 2 4

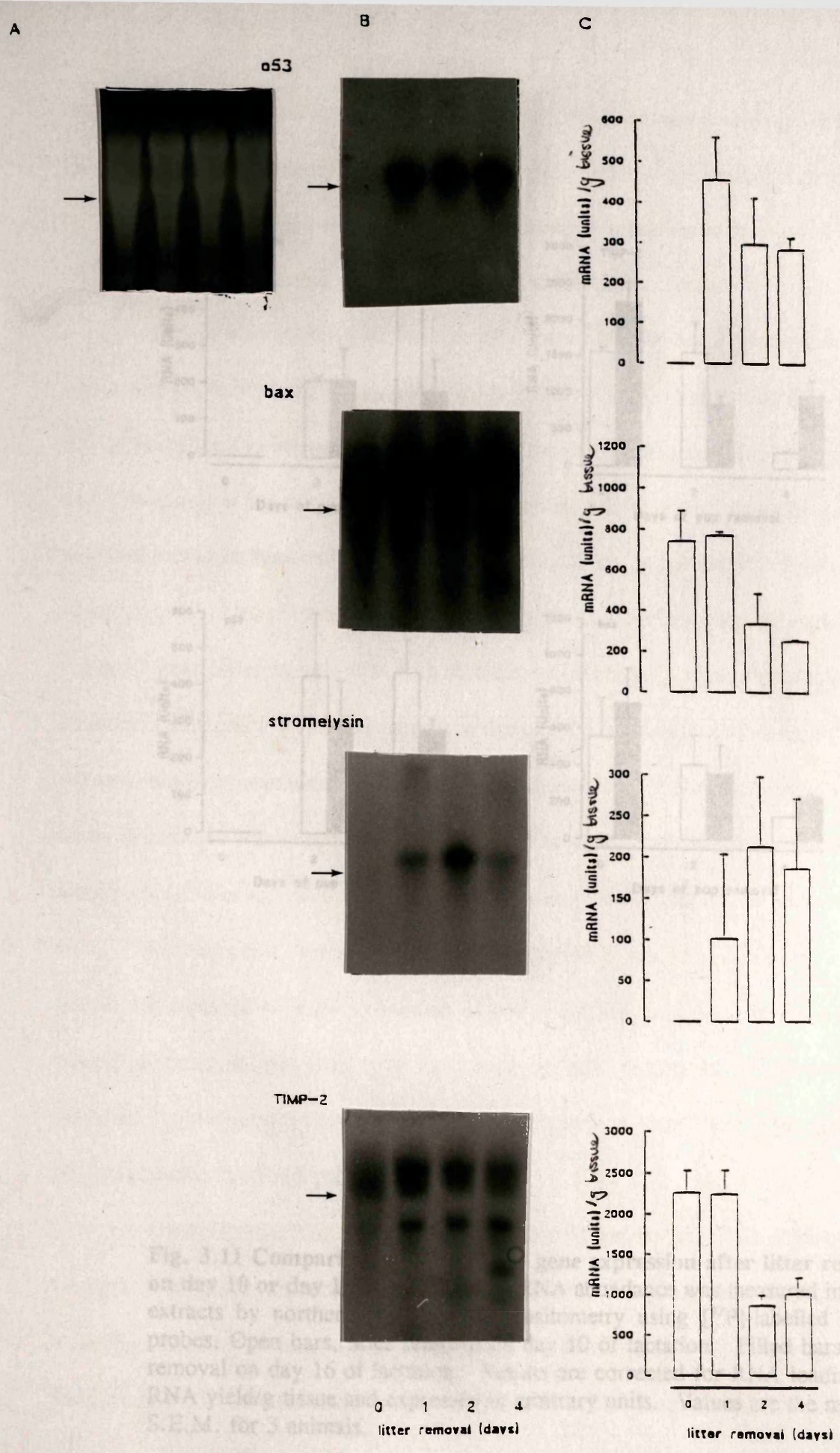
litter removal (days)

TABLE 3.2 Quantitative Analysis of mRNA levels

	RNA yield (UG /G TISSUE)		Stromelysin				TIMP-2				p53				bax			
			mRNA (units)/ RNA (ug)		mRNA (units)/ tissue (g)		mRNA (units)/ RNA (ug)		mRNA (units)/ tissue (g)		mRNA (units)/ RNA (ug)		mRNA (units)/ tissue (g)		mRNA (units)/ RNA (ug)		mRNA (units)/ tissue (g)	
	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev
Timepoint in lactation (L)																		
10L +4 days pup removal	2300	190	0.21	0.04	474	82	0.09	0.01	212	12	0.19	0.05	441	91	0.06	0.01	133	7
10L +2 days pup removal	4500	730	0.05	0	209	10	0.35	0.04	1579	420	0.95	0.04	427	189	0.09	0.03	407	160
10L	11730	820	0		0		0.13	0.02	1549	203	0		0		0.05	0.02	554	211
16L +4 days pup removal	2300	250	0.08	0.05	187	121	0.44	0.10	1008	282	0.12	0.01	286	43	0.11	0.01	253	11
16L + 2 days pup removal	3400	750	0.06	0.04	213	120	0.25	0.04	862	61	0.09	0.03	298	157	0.11	0.04	366	214
16L +1day pup removal	6500	570	0		0		0.03	0.03	2242	416	0.07	0.04	457	210	0.12	0.01	771	29
16L	12200	830	0		0		0.19	0.05	2263	377	0		0		0.06	0.01	741	265
18L	9700	1050	0		0		0.20	0.03	1896	427	0		0		0.06	0.01	630	18
20L	7800	450	0		0		0.22	0.03	1735	200	0		0		0.07	0.02	563	83
22L	6500	650	0.07	0.05	435	236	0.14	0.02	928	127	0.14	0.06	942	500	0.11	0	665	95
18L whilst 18 days pregnant	9700	790	0		0		0.18	0	1737	75	0		0		0.07	0.01	721	177
20L whilst 20 days pregnant	3300	1150	0		0		0.11	0.01	350	51	0		0		0.08	0.01	277	116
22Lwhilst second litter 1L	3200	370	0.13	0.03	416	56	0.10	0.05	320	247	0.11	0.07	346	234	0.11	0	329	33
LHS unsealed, RHS unsealed 24 hrs	12700	1160	0		0		0.16	0.01	1968	340	0		0		0.07	0.01	454	42
LHS unsealed, RHS sealed 24 hrs	4600	1520	0.1	0.02	347	81	0.22	0.02	1031	356	0.09	0.01	394	109	0.04	0.01	185	112
LHS unsealed, RHS unsealed 48 hrs	12500	860	0.11	0.01	358	35	0.16	0.04	2077	476	0		0		0.06	0.01	438	58
LHS unsealed, RHS sealed 48 hrs	4900	510	0.16	0.01	879	17	0.21	0.08	1027	332	0.16	0.02	666	71	0.09	0.02	227	49

RNA was arranged on the blots as described in Methods 2.6.7, after hybridisation and autoradiography the blot were stripped with 2 % SDS and reprobed. All 4 probes i.e *stromelysin, TIMP-2, p53 and bax* were used on each blot but not in any particular order.

Fig. 3.10 Mouse mammary gene expression after litter removal on day 16 of lactation. Expression of *p53, bax, stromelysin* and *TIMP-2* was measured by northern analysis of total RNA. A, ethidium bromide staining of total RNA after agarose gel electrophoresis. B, northern analysis of RNA hybridised with [^{32}P] dCTP end-labelled cDNA probes. C, densitometry of hybridised RNA detected by autoradiography and corrected for RNA loading and RNA yield/g tissue. Values are the mean $\pm$ S.E.M. for 3 animals. Arrows indicate 18S ribosomal RNA.


A
B
C
o53
bax
stromelysin
TIMP-2
mRNA (units) /g tissue
0
100
200
300
400
500
600
800
1000
1200
50
150
250
1500
2000
2500
3000
0 1 2 4
litter removal (days)

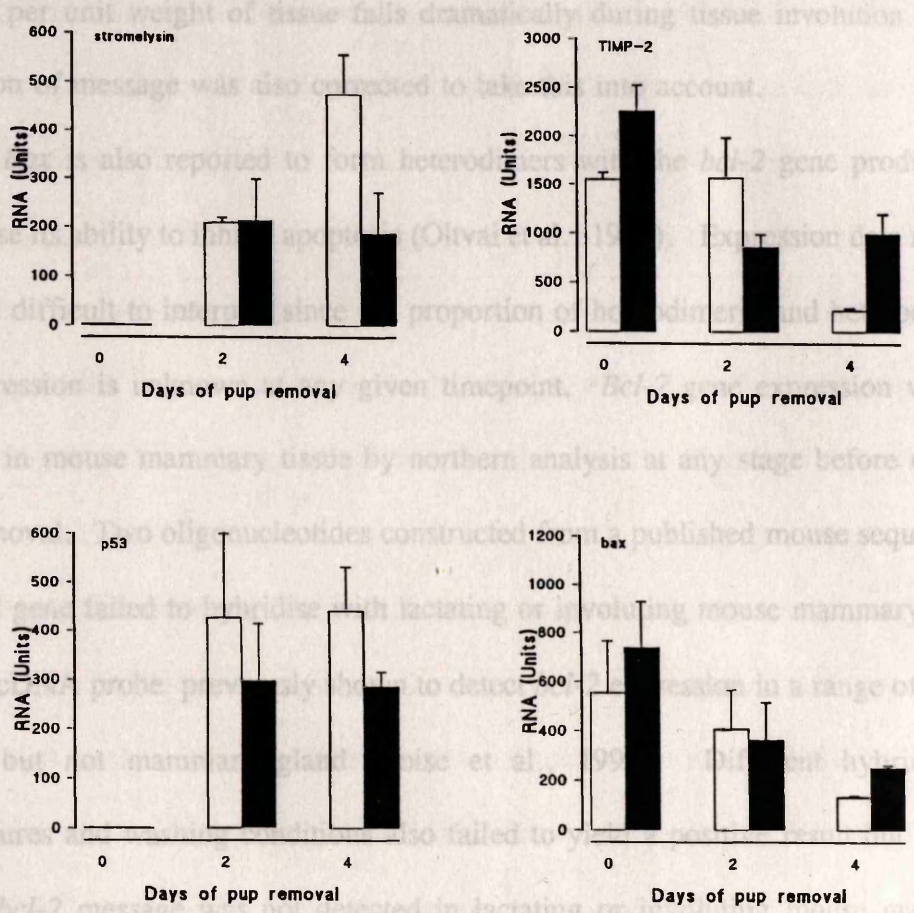


Fig. 3.11 Comparision of mammary gene expression after litter removal on day 10 or day 16 of lactation. mRNA abundance was measured in tissue extracts by northern blotting and densitometry using [^{32}P]-labelled cDNA probes. Open bars, litter removal on day 10 of lactation. Filled bars, litter removal on day 16 of lactation. Results are corrected for RNA loading and RNA yield/g tissue and expressed in arbitrary units. Values are the mean $\pm$ S.E.M. for 3 animals.

expression was induced more rapidly in late lactation. In all cases yields of RNA were normalised by densitometry to correct for differences in RNA gel loading. The amount of RNA per unit weight of tissue falls dramatically during tissue involution and so expression of message was also corrected to take this into account.

Bax is also reported to form heterodimers with the *bcl-2* gene product and antagonise its ability to inhibit apoptosis (Oltvai et al., 1993). Expression data relating to *bax* is difficult to interpret since the proportion of homodimeric and heterodimeric *bax* expression is unknown at any given timepoint. *Bcl-2* gene expression was not detected in mouse mammary tissue by northern analysis at any stage before or after litter removal. Two oligonucleotides constructed from a published mouse sequence of the *bcl-2* gene failed to hybridise with lactating or involuting mouse mammary RNA, as did a cDNA probe previously shown to detect *bcl-2* expression in a range of mouse tissues, but not mammary gland (Boise et al., 1993). Different hybridisation temperatures and washing conditions also failed to yield a positive result but in each attempt *bcl-2* message was not detected in lactating or involuting mouse mammary tissue. However, bcl-2 message was also not detected in adult mouse spleen, a tissue previously reported to show expression of bcl-2, and this suggests that none of the hybridisation conditions tried may have been optimal, despite having tried several different hybridisation temperatures, since expression was not detected with oligonucleotide or cDNA probes.

Tissue remodelling during mammary involution is the result of concerted changes in proteases and their inhibitors (Talhouk et al., 1991, 1992). These events were monitored by northern blot measurement of stromelysin mRNA, a major protease in involuting mouse mammary tissue (Talhouk et al., 1991), and that of its inhibitor,

were monitored by northern blot measurement of stromelysin mRNA, a major protease in involuting mouse mammary tissue (Talhouk et al., 1991), and that of its inhibitor, TIMP-2 (Docherty *et al.* 1985). Stromelysin RNA was undetectable in lactating tissue but increased thereafter. TIMP-2 was detected as a major band and several larger and smaller transcripts, (Figs 3.9 - 3.11) as reported previously (Gewert et al., 1987). Analysis of either the 800 bp band alone or the whole area exposed to the *TIMP-2* cDNA probe showed proportionately similar changes. In this chapter all values of *TIMP-2* expression refer to densitometry of the single 800 bp band. It was expressed at a relatively high level on day 16 of lactation and one day after litter removal, but decreased thereafter (Figs 3.9 - 3.11). This suggests maximum proteolytic activity after one day of litter removal on day 16 of lactation. Stromelysin expression was also induced rapidly by litter removal on day 10, but in this case, TIMP expression remained high on the second day of litter removal, and it was not until after 2 days of litter removal that the balance of protease to inhibitor appeared to favour degradation. Therefore, the pattern of protease and protease inhibitor expression suggested that proteolysis was induced more rapidly by litter removal in late lactation.

3.3.3. Natural weaning

In mice, natural weaning of the litter begins around day 16 of lactation. As a result, milk yield decreased from 13.8±1.3 g/day on day 16 of lactation to 0.7±0.3 on day 20, as the young became accustomed to solid food (Shipman et al., 1987). Weaning was accompanied by progressive loss of tissue organisation similar to, but slower than that observed after litter removal. On day 18, the alveoli remained intact, but by day 20 epithelial integrity was lost and alveoli had collapsed, with widespread evidence of

nuclear karyohexis (Fig 3.12).

Morphological degeneration was accompanied by mammary cell loss and an increase in DNA laddering, indicating that, as with litter removal, apoptotic death contributes to tissue involution during natural weaning (Fig 3.13). Laddering was detectable on day 16 - 18 at a level comparable to that observed in day 10-lactating mammary tissue. This increased on day 18-20, and on day 22 of lactation, despite the continuing presence of the litter, laddering had increased to a level similar to that observed after litter removal. Accordingly, tissue DNA content was unchanged on day 18, but lower by 23% and 50% than day 16 values on days 20 and 22 respectively. Programmed cell death appeared to be largely complete by day 24. At this stage DNA laddering was barely detectable (Fig 3.13), and tissue DNA content was 30% of that on day 16.

Degenerative changes in tissue morphology were associated with a progressive decline in TIMP-2 expression and, on day 22, induction of stromelysin mRNA, which had been undetectable by northern analysis up to this time (Figs 3.14 - 3.15). A high level of apoptosis on day 22 coincided with induction of *p53* mRNA, and a high level of *bax* gene transcripts (Figs 3.14 - 3.15).

Mammary involution in mice concurrently pregnant while weaning their litters differed markedly with respect to tissue morphology (Fig 3.16), time course of programmed cell death (Fig 3.17) and pattern of gene expression from that in non-pregnant animals (Figs 3.18 - 3.19). Loss of alveolar integrity was apparent on day 18 of lactation and well advanced by day 20, at which stage involution in concurrently pregnant animals resembled that induced by litter removal more than the gradual changes induced by natural weaning in non-pregnant animals. On day 22, i.e. day 1

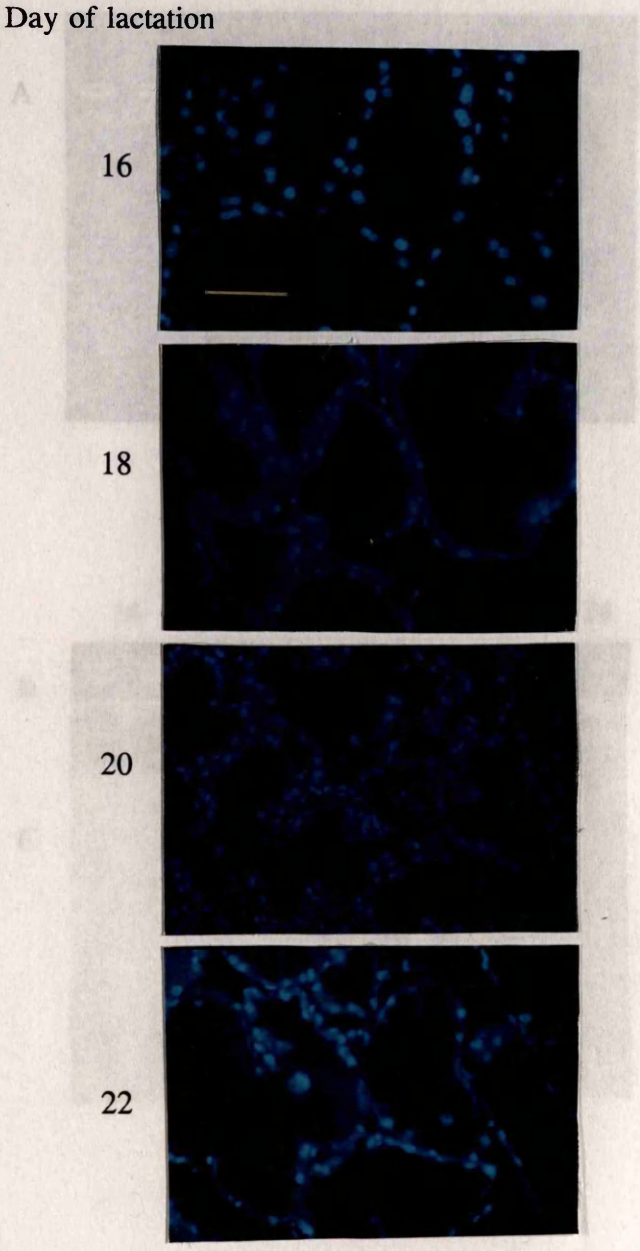


Fig 3.12 Histology of mouse mammary tissue during natural weaning. Tissue was collected *post mortem* on day 16, 18, 20 and 22 of lactation. Tissue sections were stained with DAPI. Bar = 40 μm.

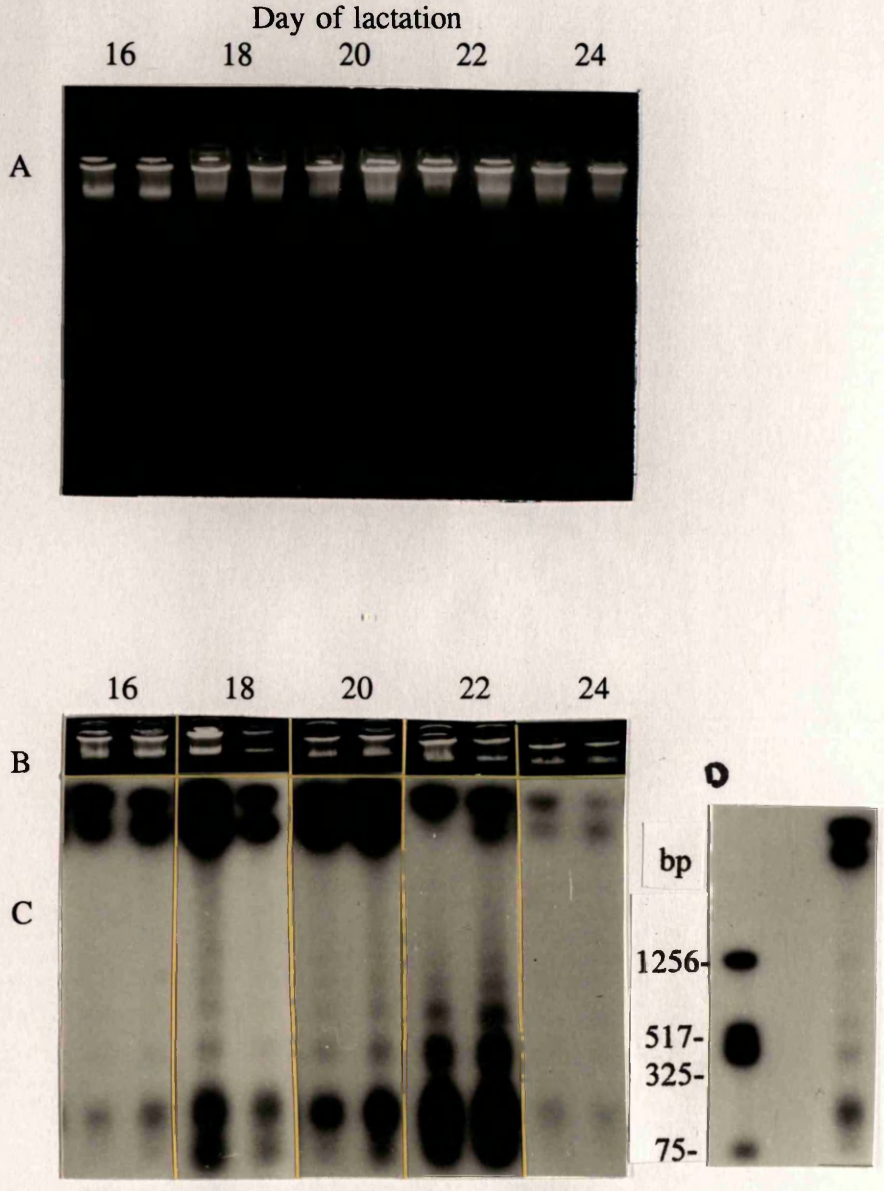
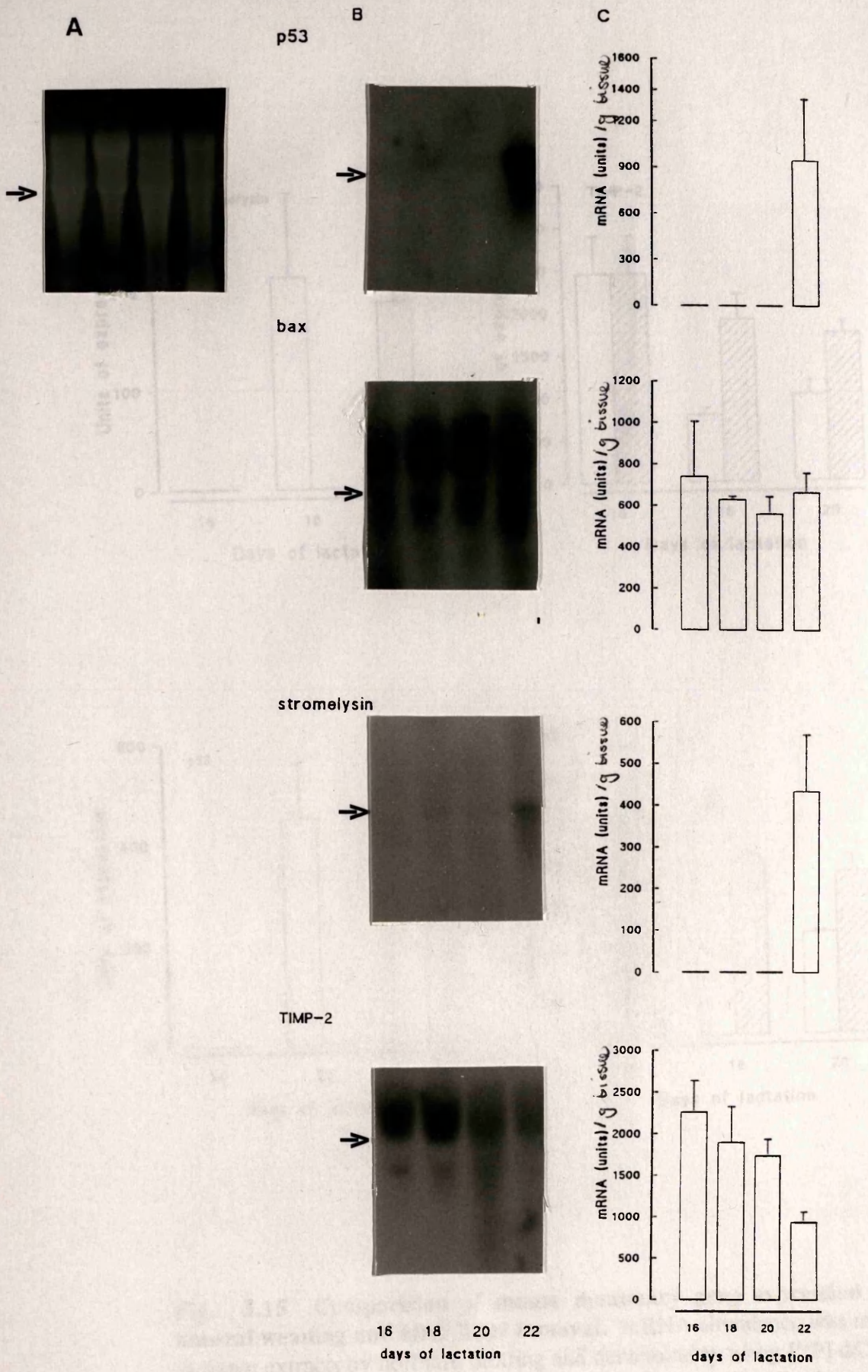


Fig. 3.13 DNA laddering in mouse mammary tissue during natural weaning. Tissue was collected *post mortem* on days 16, 18, 20, 22 and 24 of lactation. Duplicate samples are shown. A, ethidium bromide staining of resolved DNA. B, ethidium bromide staining of unresolved DNA in sample wells to confirm loading of samples . C, autoradiography of [^{32}P] dCTP end-labelled DNA. Results from two animals are shown at each timepoint.

A, 10 g DNA loaded. B, 1 g DNA loaded.

D, relative position of molecular weight markers with respect to electrophoresed DNA.

Fig. 3.14 Mouse mammary gene expression during natural weaning. Expression of *p53, bax, stromelysin* and *TIMP-2* was measured by northern analysis of total RNA. A, ethidium bromide staining of total RNA after agarose gel electrophoresis. B, northern analysis of RNA hybridised with [^{32}P] dCTP end-labelled cDNA probes. C, densitometry of hybridised RNA detected by autoradiography and corrected for RNA loading and RNA yield/g tissue. Values are the mean $\pm$ S.E.M. for 3 animals. Arrows indicate 18S RNA.


A
B
C
p53
bax
stromelysin
TIMP-2
mRNA (units) /g tissue
0
300
600
900
1200
1400
1600
200
400
800
1000
100
500
2000
2500
3000
16
18
20
22
days of lactation

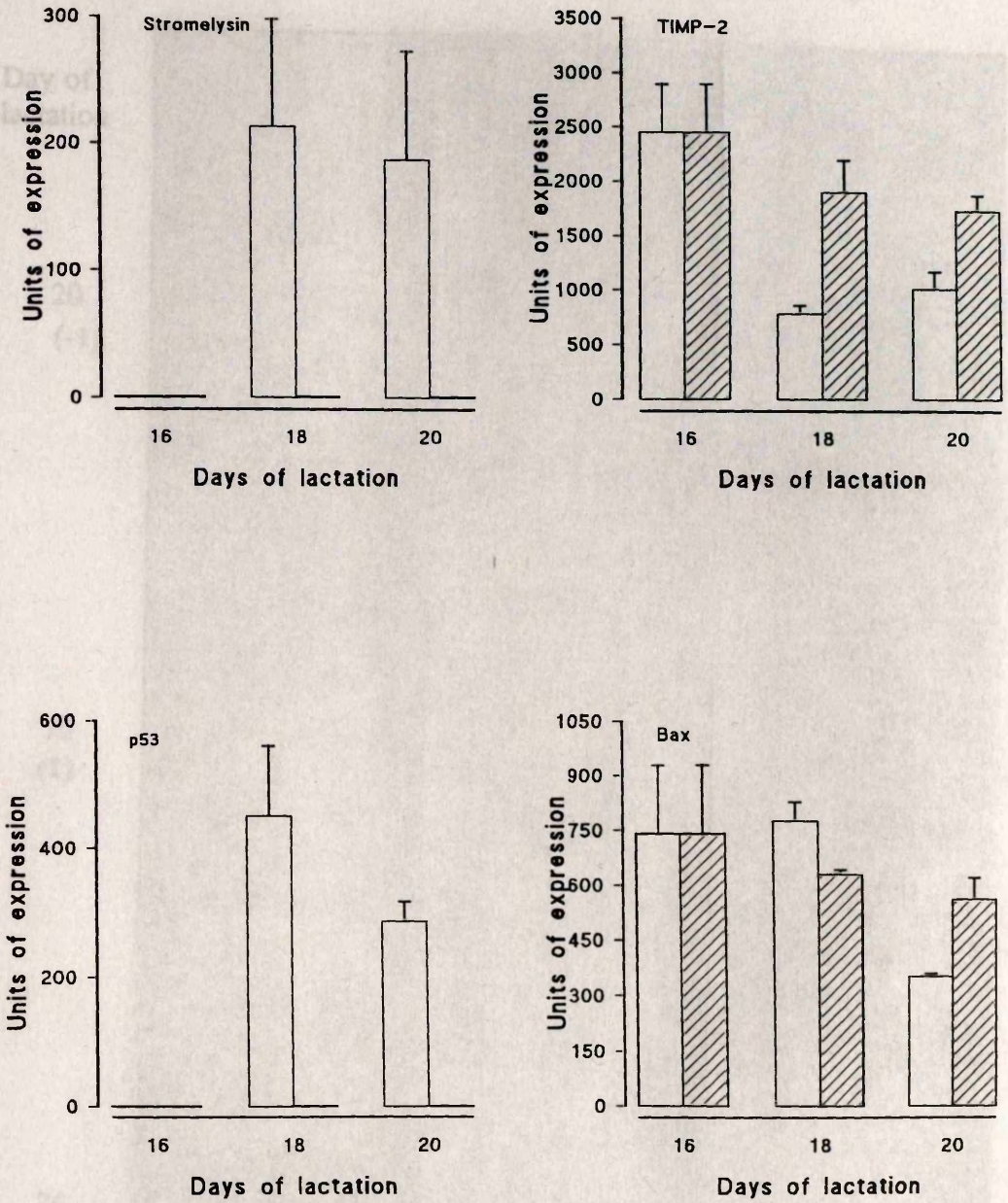


Fig. 3.15 Comparision of mouse mammary gene expression during natural weaning and after litter removal. mRNA abundance was measured in tissue extracts by northern blotting and densitometry using [^{32}P] dCTP end-labelled cDNA probes. Open bars, with pup removal. Hatched bars, without pup removal. Results are corrected for RNA yield/g tissue and expressed in arbitrary units. Values are the mean ± S.E.M. for 3 animals.

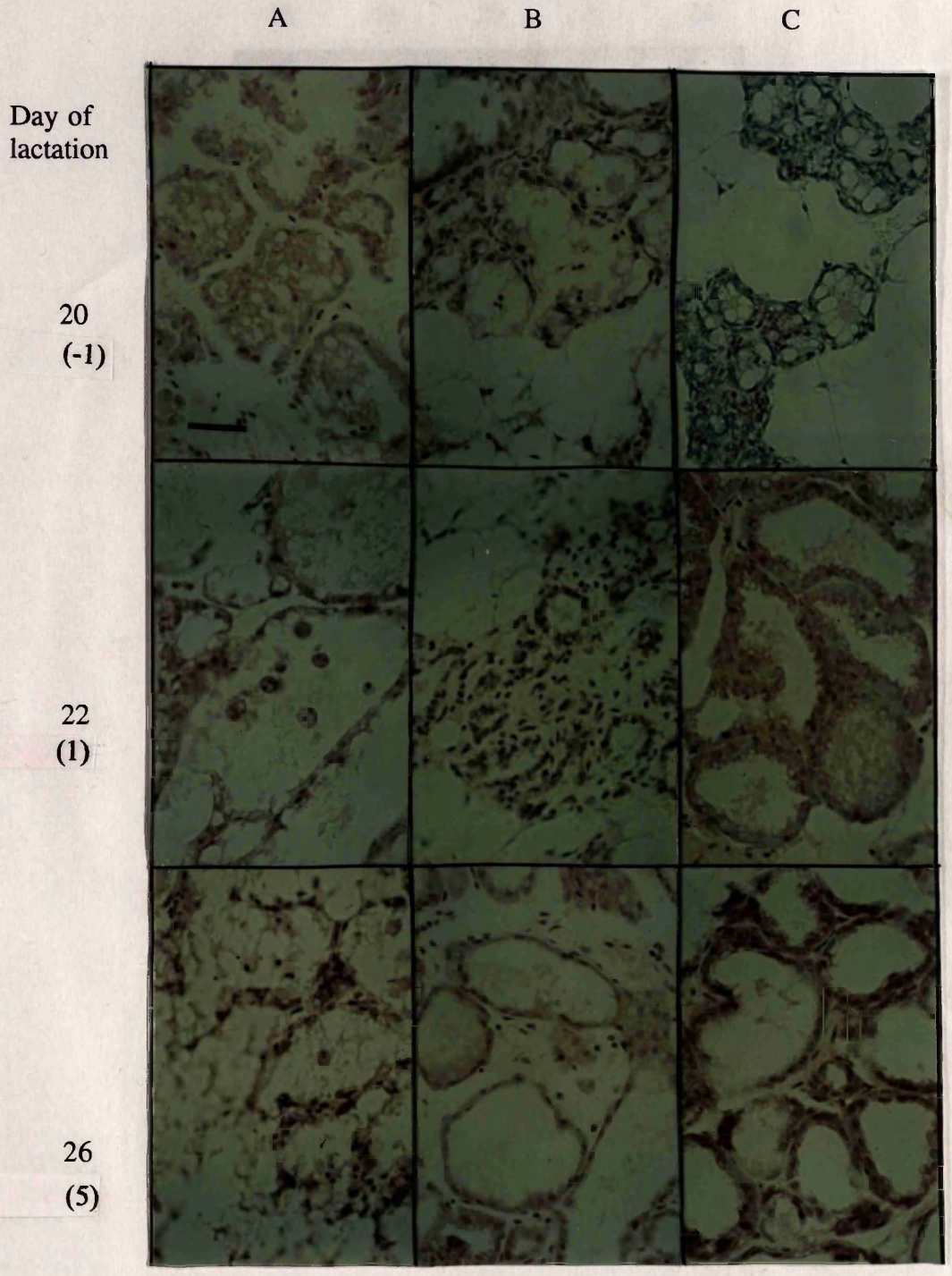


Fig. 3.16 Histology of mouse mammary tissue during natural weaning in non-pregnant and pregnant mice. Tissue was collected *post mortem* on days 20, 22 and 26 of lactation. A, non pregnant mice. B, concurrently pregnant mice; day of second lactation in parentheses. C, first pregnancy and early lactation controls.; **day of lactation in parentheses.** Bar = 40 μm. **Haemotoxylin and eosin staining.**

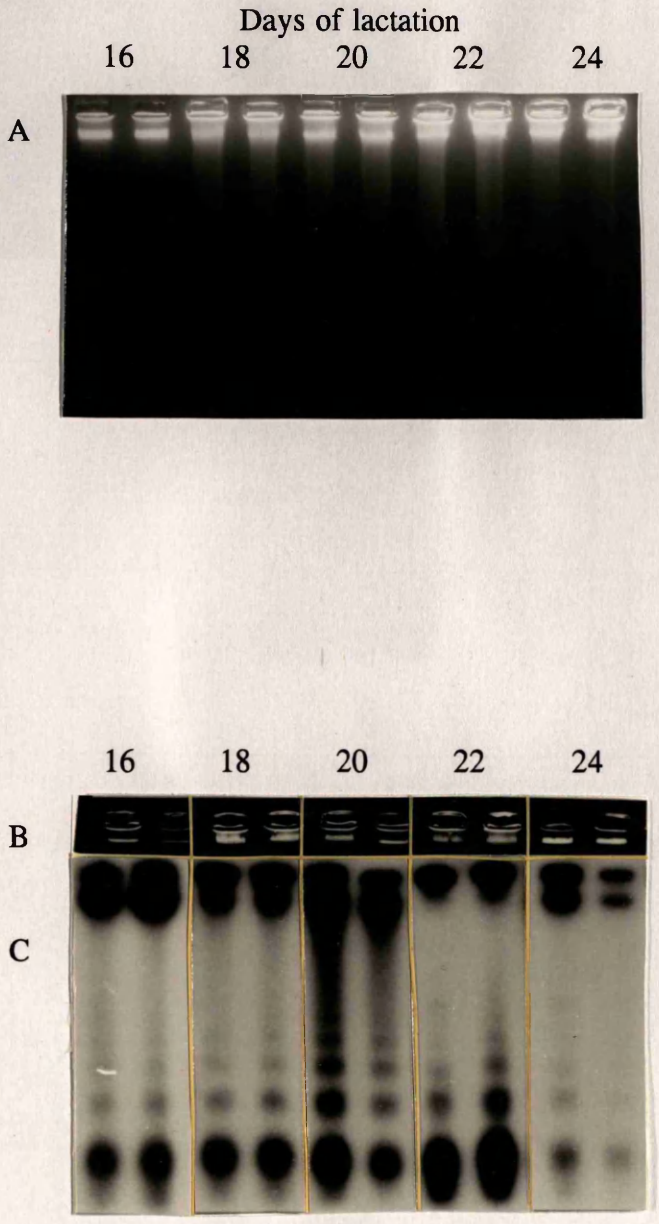
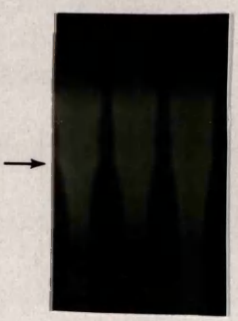


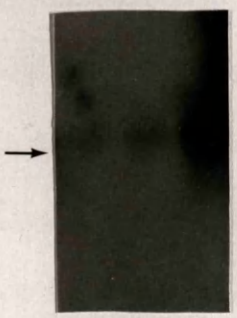
Fig. 3.17 DNA laddering during natural weaning in concurrently pregnant mice. Tissue was collected *post mortem* on day 16, 18, 20, 22 or 24 of the first lactation. Re-mated mice littered on day 21 of lactation. A, ethidium bromide staining of resolved DNA. B, ethidium bromide staining of unresolved DNA in sample wells to confirm equal loading of samples. C, autoradiography of [^{32}P] dCTP end-labelled DNA. Results from two aminals are shown at each timepoint.

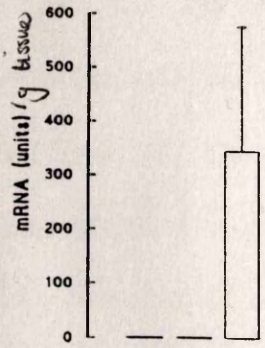
Fig. 3.18 Mammary gene expression during weaning in concurrently pregnant mice. Expression of *p53, bax, stromelysin* and *TIMP-2* was measured by northern analysis of total RNA. A, ethidium bromide staining of total RNA after agarose gel electrophoresis. B, northern analysis of RNA hybridised with [^{32}P] dCTP end-labelled cDNA probes. C, densitometry of hybridised RNA detected by autoradiography and corrected for RNA loading and RNA yield/g tissue. Values are the mean $\pm$ S.E.M. for 3 animals. Arrows indicate 18S ribosomal RNA.

A B C

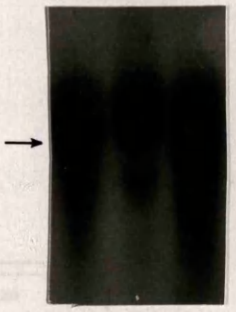
p53

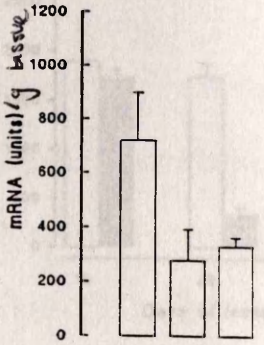




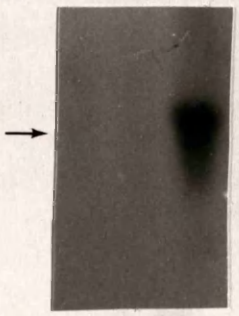


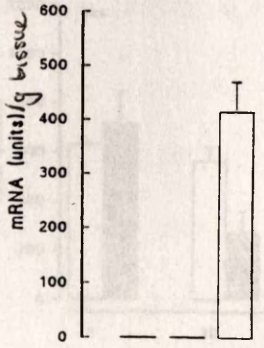
bax



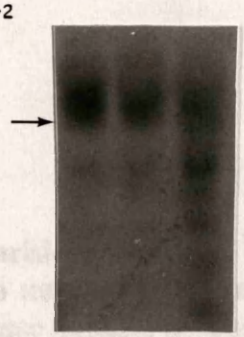


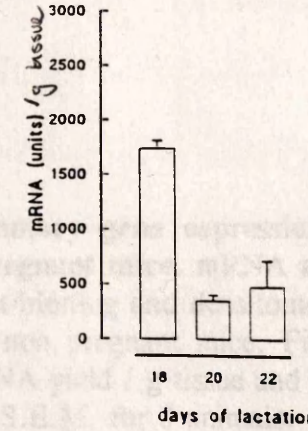
stromelysin





TIMP-2





18 20 22

days of lactation

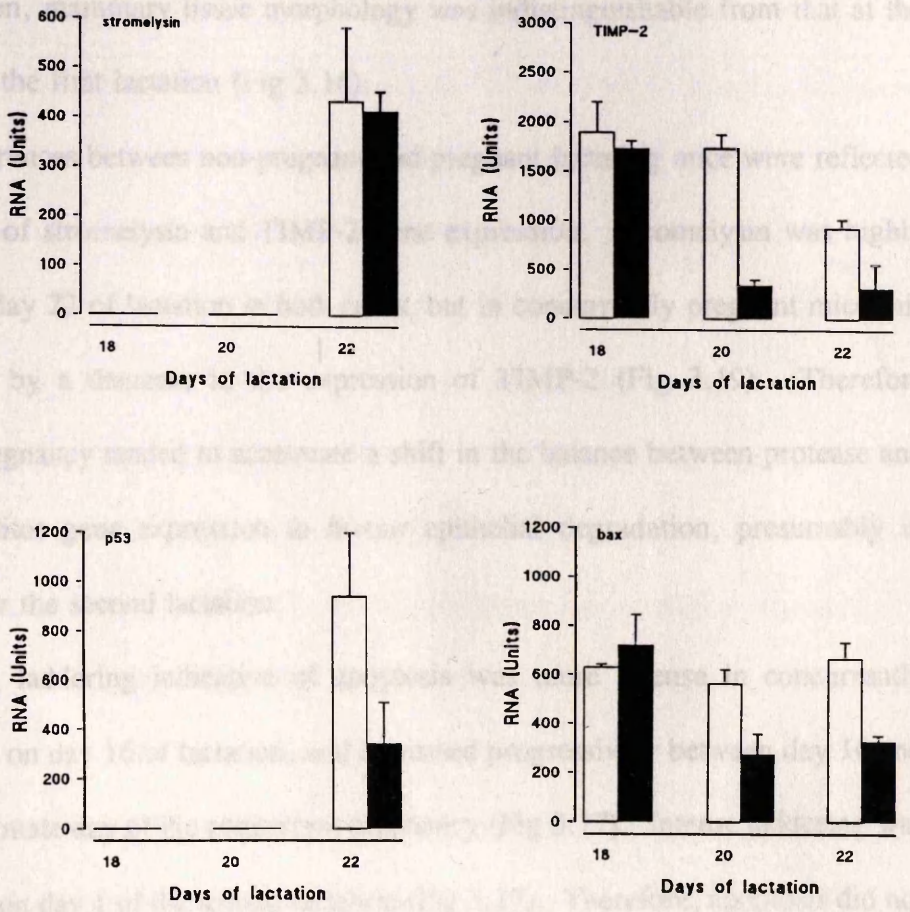


Fig. 3.19 Comparision of mouse mammary gene expression during natural weaning in non-pregnant and pregnant mice. mRNA abundance was measured in tissue extracts by northern blotting and densitometry using [^{32}P]-labelled cDNA probes. Open bars, non pregnant mice. Filled bars, remated mice. Results are corrected for RNA yield / g tissue and expressed in arbitrary units. Values are the mean ± S.E.M. for 3 animals.

of the second lactation, the glands of concurrently pregnant mice still showed little evidence of alveolar structure, and epithelial cell nuclei and total cell volume remained small compared with fully differentiated, lactating cells (Fig 3.16). Thus, the tissue resembled neither that in late involution, nor that of early lactating tissue, in which alveoli were intact and distended with accumulated milk. However, by day 5 of the second lactation, mammary tissue morphology was indistinguishable from that at the same stage of the first lactation (Fig 3.16).

Differences between non-pregnant and pregnant lactating mice were reflected in the pattern of stromelysin and TIMP-2 gene expression. Stromelysin was highly expressed on day 22 of lactation in both cases, but in concurrently pregnant mice this was preceded by a decrease in the expression of TIMP-2 (Fig 3.19). Therefore concurrent pregnancy tended to accelerate a shift in the balance between protease and protease inhibitor gene expression to favour epithelial degradation, presumably in preparation for the second lactation.

DNA laddering indicative of apoptosis was more intense in concurrently pregnant mice on day 16 of lactation, and increased progressively between day 16 and 20, the penultimate day of the concurrent pregnancy (Fig 3.17). Intense laddering was also observed on day 1 of the second lactation (Fig 3.17). Therefore, apoptosis did not cease with parturition; as suggested by the tissue's morphology, remodelling of the mammary glands continued during the early days of the second lactation. However, by day 5 of the second lactation, laddering was detected at a level similar to that observed in established lactation (Fig 3.17). Northern analysis detected *p53* expression in mammary tissue of concurrently pregnant mice only on day 1 of their second lactation, i.e. coincident with the most intense DNA laddering. *p53* is also expressed

Table 3.2
on day 22 of lactation in non pregnant mice (Figs 3.18 - 3.19). However, in concurrently pregnant mice, *p53* mRNA expression was preceded by a decrease in *bax* mRNA abundance (Figs 3.18 -3.19).
Table 3.2

3.3.4. Milk stasis

Milk stasis was induced unilaterally by teat sealing one body half, while allowing the dam to suckle a smaller litter from the other glands. Macroscopic tissue examination within 24 h of teat sealing showed milk accumulation at a whole gland level (Fig 3.20), and histological examination of teat sealed glands after 24 h treatment showed alveoli were distended with milk (Fig 3.21). After 48 h, distension had decreased, and the secretory epithelium showed evidence of disorganisation similar to that observed after litter removal at the same stage of lactation (Fig 3.21). Tissue from unsealed glands after 24 h was morphologically similar to that of 10 day lactating mice, however by 48 h of teat sealing in the contralateral gland some evidence for involution was apparent in the suckled gland, showing that the induction of involution occurred preferentially but not exclusively in teat sealed glands (Fig 3.21).

The low level DNA laddering observed in day 10 lactating mouse mammary tissue increased progressively with 24 h and 48 h of milk stasis (Fig 3.22). The intensity of laddering was comparable to that observed after litter removal, suggesting that milk stasis was highly effective in inducing mammary apoptosis. Laddering also increased, albeit to a lesser degree, in contralateral glands suckled by the remaining pups (Fig 3.22).

Milk stasis produced ipsilateral changes in stromelysin and TIMP-2 gene expression (Figs 3.23). Whereas stromelysin mRNA was undetectable in day 10
Table 3.2

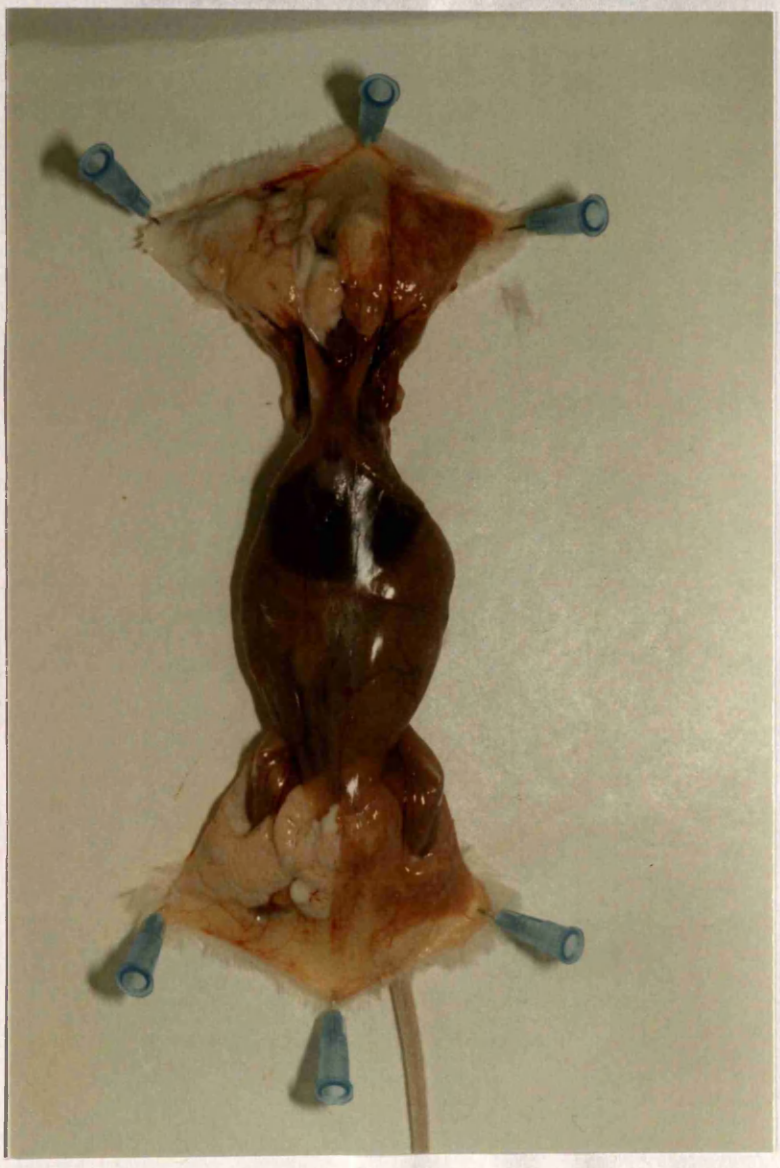


Fig. 3.20 Macroscopic appearance of mouse mammary tissue after unilateral teat sealing. Right-side teats were sealed on day 10 of lactation and the animal was killed by cervical dislocation 24 h later.

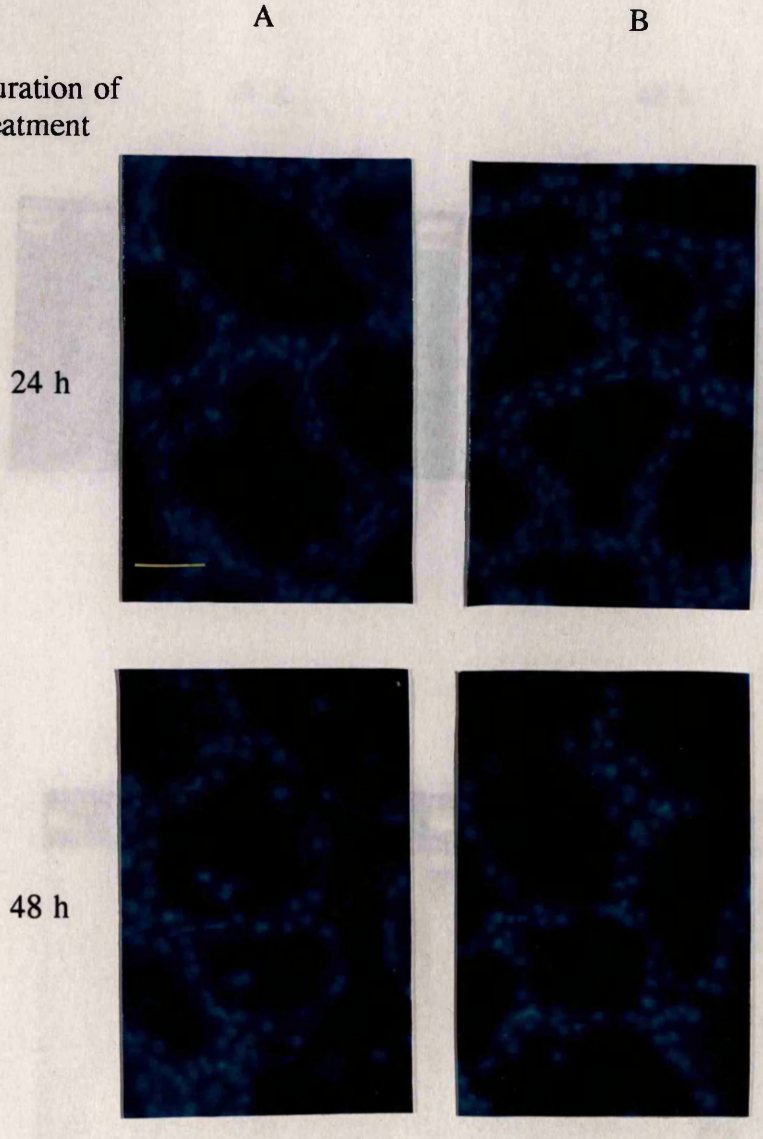


Fig. 3.21 Histology of mouse mammary tissue after unilateral teat sealing. A, left side suckled by a reduced number of pups for 24 h and 48 h. B, right-side teats were sealed on day 10 of lactation for 24 h and 48 h prior to tissue collection. Tissue sections were stained with DAPI. Bar = 40 μm.

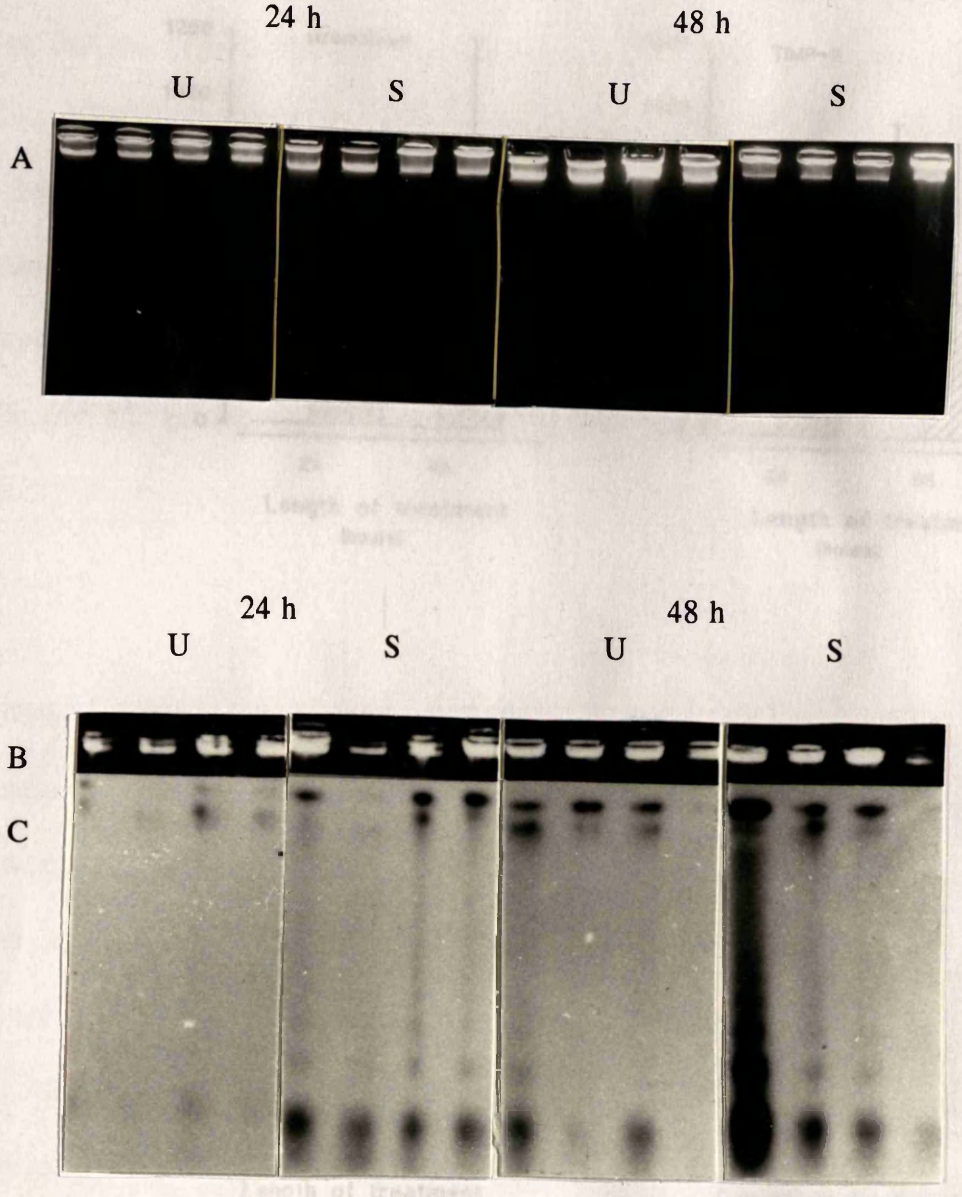


Fig. 3.22 DNA laddering after unilateral teat sealing of mouse mammary glands in mid-lactation. Tissue was collected *post mortem* from sealed (S) and unsealed (U) mammary glands 24 h or 48 h after sealing on day 10 of lactation. Four animals are shown at each time point. A, ethidium bromide staining of resolved DNA. B, ethidium bromide staining of unresolved DNA in sample wells to confirm equal loading of samples. C, autoradiography of [^{32}P] dCTP end-labelled DNA.

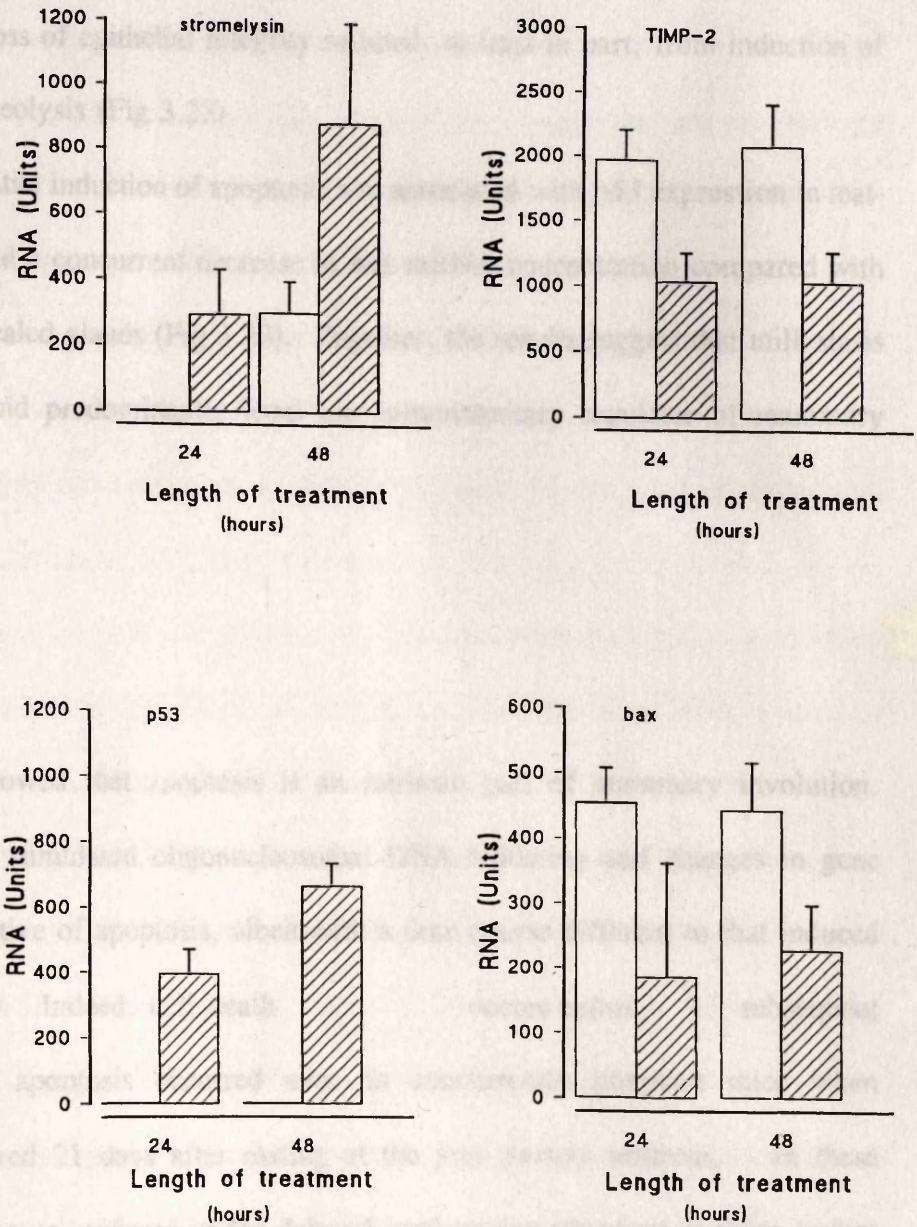


Fig. 3.23 Comparision of gene expression induced by unilateral milk stasis at mid-lactation. Unilateral milk stasis was induced by teat-sealing half the mammary glands on day 10 of lactation. Tissue was collected *post mortem* 24 h and 48 h later. mRNA abundance was measured by densitometry after northern blotting and autoradiography using [^{32}P]-labelled probes. Results are corrected for RNA loading and yield/g tissue, and are the mean $\pm$ S.E.M. for 3 animals. Open bars, unsealed glands. Hatched bars, teat sealed glands.

lactating glands and in unsealed glands, its expression was increased by 48 h of milk stasis. This was accompanied by an ipsilateral decrease in TIMP-2 expression, suggesting that loss of epithelial integrity resulted, at least in part, from induction of extracellular proteolysis (Fig 3.23).

Preferential induction of apoptosis was associated with *p53* expression in teat-sealed glands, and a concurrent decrease in *bax* mRNA concentration compared with contralateral unsealed glands (Fig 3.23). Together, the results suggest that milk stasis is an effective and predominantly local i.e. intramammary regulator of mammary apoptosis.

3.4 Discussion

These studies showed that apoptosis is an intrinsic part of mammary involution. Natural weaning stimulated oligonucleosomal DNA laddering and changes in gene expression indicative of apoptosis, albeit with a time course different to that induced by litter removal. Indeed, cell death occurs before subsequent lactations, since apoptosis occurred even in concurrently pregnant mice when parturition occurred 21 days after mating at the *post partum* oestrous. In these animals, there was no evidence of the delayed implantation observed in other studies (C.H. Knight, personal communication) and no intervening dry period between lactations. To accommodate a period of involution, it was clear that mammary apoptosis was induced earlier in concurrently pregnant mice, and to an extent comparable with that found after litter removal. Mammary histology and the detection of DNA laddering on day one of the second lactation suggested that involution and

mammary re-development may have proceeded simultaneously in pregnant, lactating mice. A similar observation was made in lactating goats, which were found to be capable of lactogenesis and successful lactation without an intervening dry period (Knight and Wilde, 1988). In concurrently pregnant goats, mammary cell proliferation increased late in the first lactation, suggesting that milk production in the second was at least in part from new epithelial cells (Knight and Wilde, 1988). However, in those goats, as in other species including the dairy cow (Whittemore, 1980), milk yield in late lactation declined faster than in non pregnant controls, perhaps as a result of increased circulating concentrations of oestrogens which, on the basis of the present study, may have stimulated mammary apoptosis.

Accelerated apoptosis in concurrently pregnant mice may reflect a maternal decision to discontinue investment in the weanling offspring, and to invest instead in the next litter. Parent-offspring conflict arises because the long term interests of the parent are not identical with those of the offspring (Trivers, 1974). The parent's long term reproductive success may be better served by premature weaning, since it would allow her to replenish body reserves or as in the present study, to partition nutrients towards the litter *in utero*. Triver's cost benefit analysis showed that it is the mother who sets the overall strategy for parental investment. When nutrition is not limited and breeding does not have to be fitted into a tight reproductive window, the outcome is in favour of the young. When this is not the case, the outcome of the investment analysis is more complicated. Species with a long gestation or lactation may, even under adverse conditions, continue investment in young whereas, as suggested by the present study, rodents have little incentive to do so (Peaker, 1989).

Natural weaning, both in non-pregnant and concurrently-pregnant mice,

occurred without any evidence of alveolar distension caused by milk stasis, indicating that the gradual nature of the process had allowed autocrine control to match the rate of milk secretion progressively to the pup's falling demand for milk (Wilde et al., 1995b). This distinguishes natural weaning from litter removal, when induction of apoptosis is likely to be a consequence not just of falling galactopoietic hormone concentrations (Sheffield and Kotolski, 1992; Atwood et al., 1995), but of milk accumulation in the glands. Teat sealing clearly identified milk stasis as an effective local, i.e. intra-mammary inducer of mammary apoptosis. Whether this mechanism is chemical in nature, or due to the physical presence of stored milk is not known. However, the tissue is both a source and the target of a number of growth factors, some of which may regulate cell death as well as cell proliferation. For example, an increase in transforming growth factor ß1 (TGFß1) and TGFß3 expression during involution *in vivo* and in whole organ mammary cultures (Atwood et al., 1995), the ability of TGFß1 and TGFß3 to regulate mammary cell proliferation in non-pregnant, non-lactating animals (Robinson et al., 1991), and the secretion of TGF-ßs in milk (Letterio et al., 1994) has led to suggestions that TGF-ß may regulate cell number during mammary involution (Atwood et al., 1995). Another possibility is that local i.e. intramammary depletion of insulin-like growth factor (IGF-1), a potent mammary mitogen (McGrath et al., 1991) and a putative cell survival factor in other tissues (Sell et al., 1995) may precipitate death. The IGF-1 binding protein (IGFBP) level in milk has been shown to increase after litter removal or galactopoietic hormone ablation to an extent which correlated well with the degree of involution induced by these treatments (Tonner et al., 1995). A further possibility is that mammary cell number is under autocrine control by the same FIL protein (Wilde et al., 1995a) which

regulates milk secretion acutely and mammary cell differentiation thereafter (Wilde et al., 1987a, 1991).

Apoptosis induced by litter removal has been reported to coincide with alveolar degeneration and an increase in extracellular proteolytic activity (Strange et al., 1992; Talhouk et al., 1992), raising suggestions that, as in other tissues (see for example, Oberhammer et al., 1991), mammary apoptosis is dependent on loss of basement membrane integrity. Accordingly, reconstituted basement membrane suppressed apoptosis in a mammary cell line, an effect counteracted by ß1 integrin antibodies, whereas over-expression of stromelysin under the whey acidic protein promoter induced mammary apoptosis in late pregnant transgenic mice (Boudreau et al., 1995). In the present study, degenerative changes in the mammary epithelium and reciprocal changes in protease and protease inhibitor expression were generally supportive of a causal relationship between basement membrane integrity and apoptosis. However, it was notable that appearance of DNA laddering during natural weaning preceded, in both non-pregnant and concurrently pregnant mice, any change in stromelysin or TIMP-2 mRNA. It is of course, possible that other protease activities are induced more rapidly than that of stromelysin e.g. gelatinase (Talhouk et al., 1992). On the other hand, nick end labelling of fragmented DNA also detected apoptosis in fully lactating tissue when alveolar epithelium was apparently intact. Therefore, regulation of apoptosis may be more subtle, or more localised, than might be inferred from the widespread tissue degeneration that constitutes mammary involution. Localised basement membrane degradation in otherwise intact alveoli may stimulate apoptosis of individual cells, while neighbouring cells are protected by local production of protease inhibitors (Alexander and Werb, 1991; Saskela and Rifkin,

1988).

As observed previously (Strange et al., 1992), mammary apoptosis was consistently associated with an increase in *p53* mRNA abundance, indicating that it provides a useful indicator of cell death in this, as in other tissues. *p53* plays a key role in the regulation of cell proliferation and apoptosis, in that the gene product causes G1/S phase arrest (Diller et al., 1990; Michalovitz et al., 1990), allowing DNA repair or, alternatively, initiation of apoptosis (Kastan et al., 1991). Mutation of the *p53* gene, or an altered level of p53 protein was frequently observed in mammary hyperplastic alveolar nodules (Jerry et al., 1993), the most common neoplastic mammary lesion in rodents (Medina, 1988). *p53*- mediated regulation of apoptosis is modified by a number of genes, one of which is *bax,* a member of the *bcl-2* proto-oncogene family (Wang et al., 1993; Chiou et al., 1994). *Bax* in turn, may be a *p53* primary response gene (Miyashita et al., 1994; Miyashita and Reed, 1995). Its role in apoptosis is not yet fully elucidated, but the *bax* gene product may either form homodimers and itself stimulate apoptosis, or alternatively may form heterodimers with the Bcl-2 protein, thereby inhibiting its suppression of apoptosis (Oltvai et al., 1993). The circumstances dictating whether *bax* stimulates or inhibits apoptosis are not yet well defined, and so the contrasting effects of *bax* on litter removal and natural weaning on the one hand, and pregnancy-accelerated weaning and milk stasis on the other, are not easily rationalised. This is particularly the case when northern analysis failed to detect *bcl-2* gene expression at any stage of murine lactation or involution. The Bcl-2 protein is an inhibitor of apoptosis (Vaux *et al*, 1992; Garcia et al., 1992), whereas decreased levels of the protein can render cells more susceptible to apoptotic death (Miyashita and Reed, 1993). The absence of *bcl-2* gene expression in lactating

or involuting rodent tissue suggests that this protein is not itself a regulator of mammary apoptosis. On the other hand, cyclical changes in Bcl-2 protein in human tissue during the oestrous cycle suggest that it may mediate the ovarian control of mammary cell number (Sabourin et al., 1994). Edwards et al., (1995) have also reported Bcl-2 and Bax protein expression in cultured mammary cells. They reported that both Bcl-2 and Bax levels were elevated when cells were cultured without basement membrane, a condition which they reported to induce apoptosis. Whatever the role of Bcl-2 in mammary apoptosis failure to detect it under any condition may reflect more the insensitivity of northern analysis than the gene product's unimportance in mammary apoptosis.

In conclusion, this study demonstrates that programmed cell death is part of the normal repertoire of the lactating and involuting mouse mammary gland. Apoptosis was induced not only by abrupt litter removal, but also during natural weaning, although the time course and extent of cell death, and its relation to other degradative events in mammary involution depended on the nature of the stimulus and the reproductive state of the animals. These studies also demonstrate a local control mechanism able in some instances over-ride the endocrine stimuli which signal life and death to a cell. In the following chapters local and endocrine regulation of apoptosis will be more fully explored.

CHAPTER FOUR

HORMONAL REGULATION OF APOPTOSIS

4.1 Introduction

In the preceding chapter we showed that apoptosis is an essential part of mammary involution and remodelling between lactations in rodents. When involution was induced naturally, by weaning, or experimentally, either by litter removal or teat sealing, the level of apoptosis increased within the gland.

During involution the cyclical endocrine changes associated with suckling cease and levels of circulating prolactin fall (Ota et al., 1962; Griffith and Turner, 1962; Jones, 1967; Richards and Benson, 1971), to result in involution of lobuloalveolar structure. Therefore, in rodents, prolactin has long been recognised as a major hormone concerned with lactogenesis and galactopoiesis. Studies have also demonstrated that prolactin can maintain lactation and prevent involution by regulation of mammary apoptosis (Sheffield and Kotolski, 1992; Atwood et al., 1995).

A role for growth hormone in the regulation of rodent mammary tissue has been identified, as until recently the role of growth hormone in maintaining rodent mammary structure and function was not clear. Increased growth hormone levels resulted in premature morphological development and functional differentiation (Bchini et al., 1991) whereas neutralisation of growth hormonal activity, by injection of specific antiserum, causes a 20% reduction in milk yield at peak lactation in rats (Flint et al., 1992). From these and other studies it appears that growth hormone is

important in establishing lactation and in maintaining maximum milk yield in rodents (Madon et al., 1986; Barber et al., 1992; Flint et al., 1992).

In this study, performed in lactating rats, we investigated the relative importance of prolactin and growth hormone in regulation of mammary apoptosis.

4.2 Experimental protocol

Three groups of rats were treated from day 12 of lactation. In one group, involution was induced by litter removal. Mammary tissue was collected *post mortem* on day 13 or 14 of lactation.

Hormone neutralisation was studied in the second group of rats. Prolactin or growth hormone were removed by injection of bromocriptine (500 μg/injection) or anti-growth hormone serum (150 mg/injection), respectively. Other rats were treated with both bromocriptine and anti-growth hormone serum. Treatment was administered twice daily and mammary tissue was collected on day 13 or 14 of lactation.

The effect of hormone replacement after 24 h of prolactin and growth hormone removal was studied in a third group of rats. Lactogenic hormones were replaced on day 13 of lactation by injection of prolactin (500 μg/injection) or recombinant bovine growth hormone (500 μg/injection; Monsanto plc, St Louis) or both. Tissue was collected on day 14 of lactation. Control mammary tissue was collected from lactating rats on day 14 of lactation.

Litters were weighed daily and milk yield was calculated by a modification of the method of Sampson and Jansen (1984).

4.3 Results

4.3.1 Lactating tissue

Milk secretion rate in day 14 lactating rats was 41 ml/d. Rat mammary tissue on day 14 of lactation was indistinguishable histologically from mouse mammary tissue at peak lactation (Fig 4.1A). Tissue appeared well organised, with large, evenly sized alveoli which contained milk but were not distended, as expected in glands suckled frequently.

In lactating rat mammary tissue, faint DNA ladders were revealed using [^{32}P] dCTP end-labelling of genomic DNA (Fig 4.2A). The intensity of laddering was similar to that previously detected in lactating mouse mammary tissue using the same method.

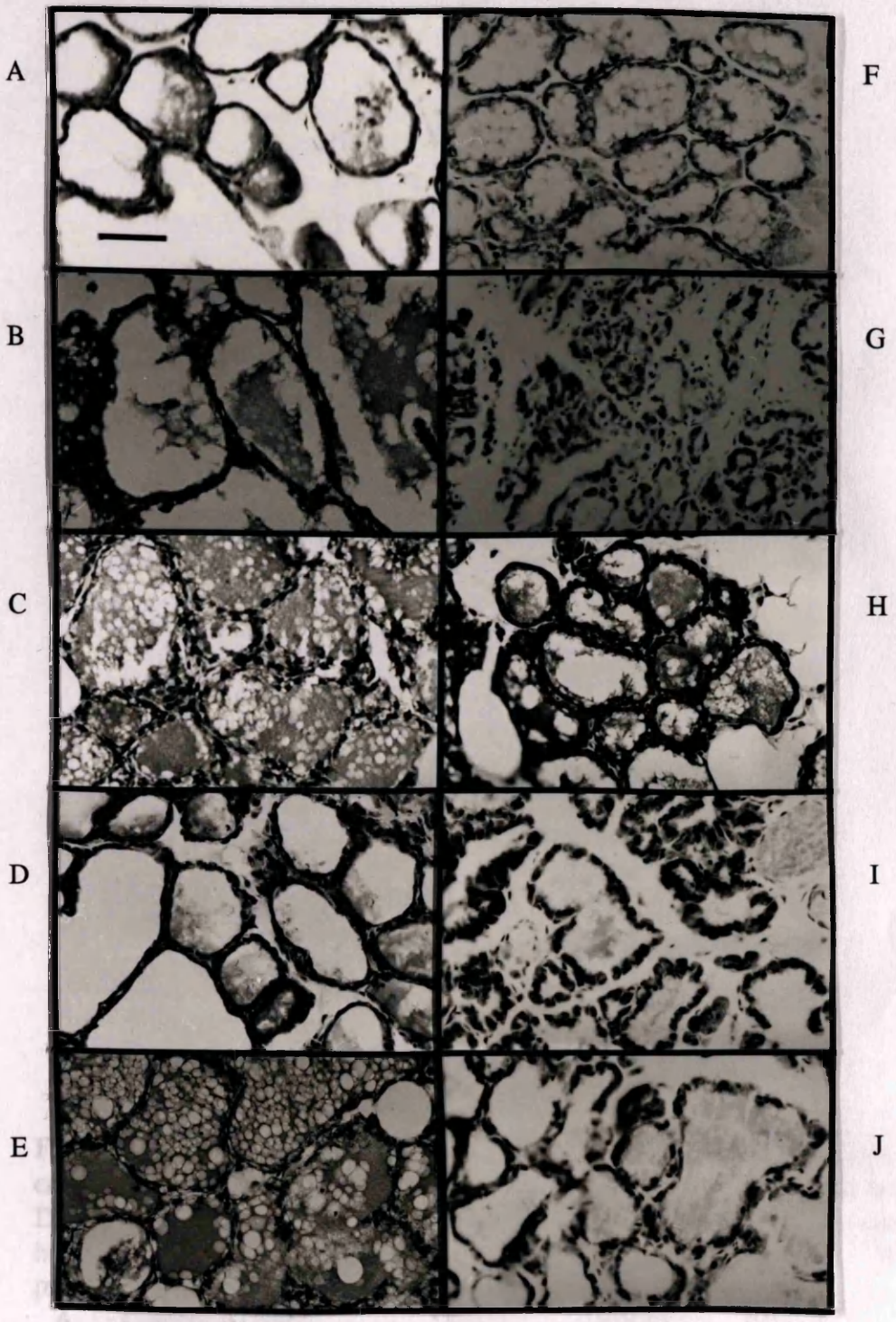
4.3.2 Litter removal

After 24 h of litter removal, alveoli were large and distended with secretion, compared with lactating rat mammary tissue (Fig 4.1B). By 48 h, alveoli were no longer distended, they appeared rounded and evenly spaced but there was evidence of milk stasis or of some secretion within alveolar spaces (Fig 4.1C). DAPI staining after litter removal showed crescent-shaped arrangements of nuclear chromatin (Fig 4.3), a phenomenon characteristic of an early phase of commitment to apoptotic death prior to endonuclease activation (Wyllie et al., 1980), and one which was not observed in mouse mammary tissue.

Litter removal produced strong DNA laddering within the first 24 h. Laddering was increased in intensity after the second 24 h period of litter removal (Fig

Fig. 4.1 Histology of rat mammary tissue. Rat mammary tissue was collected *post mortem* after various hormonal treatments, as outlined below. Sections were stained with haematoxylin and eosin. AGS, anti growth hormone serum, BR, bromocriptine, GH, growth hormone, PRL, prolactin. Bar = 40 μm. Treatments commenced on day 12 or 13 of lactation and all tissue was collected on day 14 of lactation.

A Control
B, One day, litter removal
C, Two days, litter removal
D, One day AGS
E, Two days BR
F, One day AGS + BR
G, Two days AGS + BR
H, Two days AGS + BR including one day GH
I, Two days AGS + BR including one day PRL
J, Two days AGS + BR including one day GH + PRL


A
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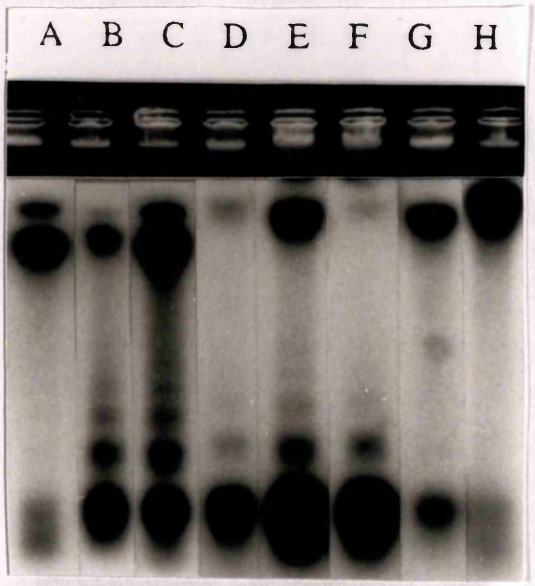


Fig. 4.2 DNA laddering of rat mammary tissue. Rat mammary tissue was collected *post mortem* after various hormonal treatments as outlined below. DNA was [^{32}P] dCTP end labelled prior to electrophoresis. AGS, anti growth hormone serum. BR, bromocriptine. GH, growth hormone, PRL, prolactin.

A, Control tisssue
B, One day of litter removal
C, Two days of litter removal
D, One day AGS + BR treatment
E, Two days AGS + BR treatment
F, One day AGS + BR followed by one day GH
G, One day AGS + BR followed by one day PRL
H, One day AGS + BR followed by one day GH + PR

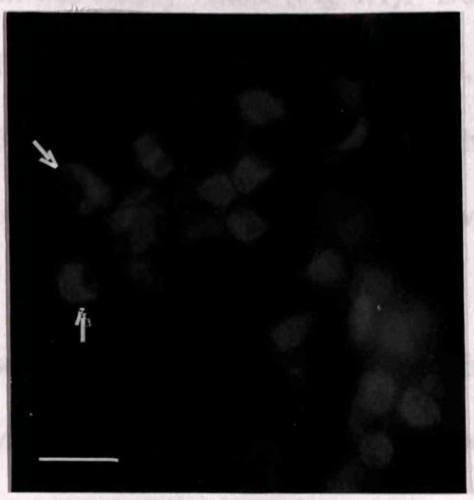


Fig. 4.3 Evidence for early changes characteristic of apoptosis in rat mammary tissue. Tissue was collected from the dam, *post mortem*, after 24 h of litter removal on day 12 of lactation. Sections were stained with DAPI. Arrows, cresented nuclei. Bar = 5 μm.

4.2).

4.3.3. Hormone neutralisation

Milk secretion rate after 48 h prolactin and growth hormone deficiency was 8 ml/d, which represented an 80% reduction compared with untreated controls. Simultaneous removal of prolactin and growth hormone resulted in morphological changes similar to involution, and after 48 h of dual hormone depletion epithelial cell loss was apparent and adipose tissue infiltration was clearly visible (Fig 4.1). During this period DNA content decreased by 37% (D.J. Flint, personal communication), a decrease which was attributable to apoptosis. DNA laddering increased within 24 h in hormone depleted rats, and after 48 h ladder intensity was greater than that observed with litter removal (Fig 4.2).

Milk yield was also reduced after removal of prolactin or growth hormone alone. Treatment for 48 h with anti-growth hormone serum reduced milk yield by 15% while treatment with bromocriptine for 48 h reduced milk yield by 50%. Therefore, single hormone removal reduces milk yield but not to the same extent as after depletion of both growth hormone and prolactin. DNA laddering and histology after 24 h growth hormone removal remained indistinguishable from control lactating tissue (Fig 4.1, 4.4). DNA ladders were slightly more prominent after 24 h prolactin removal than in control lactating tissue (Fig 4.4) and alveoli were engorged with a secretion which had a higher lipid content than milk from control animals (D. Flint, personal communication; Fig 4.1).

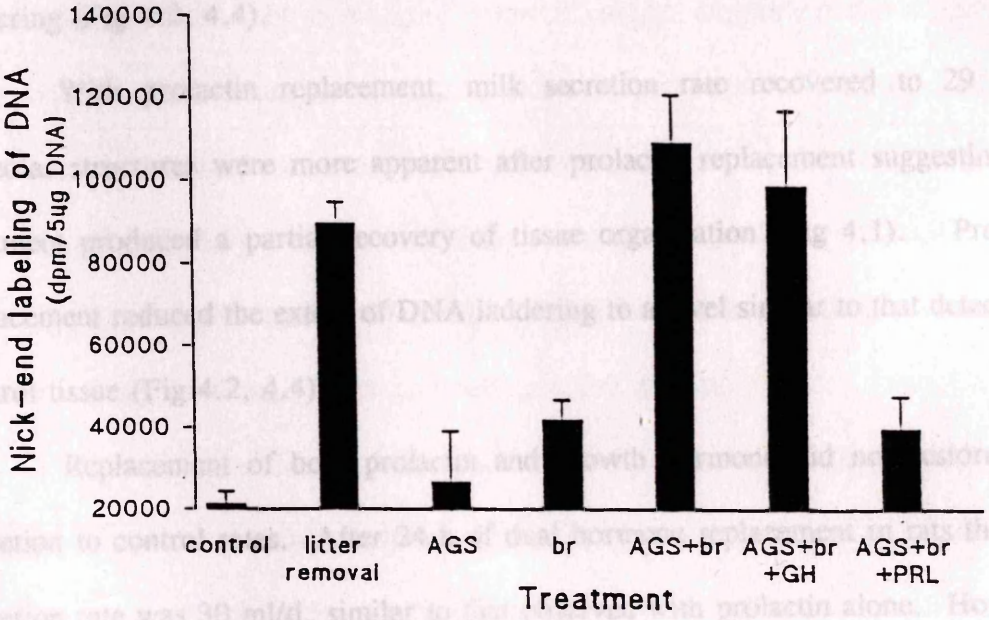


Figure 4.4 Nick end-labelling of rat mammary DNA. DNA was [^{32}P] dCTP end-labelled after various hormonal treatments as outlined below. AGS, anti growth hormone serum. br, bromocriptine, GH, growth hormone. PRL, prolactin.

Control - day 14 of lactation
Litter removal - 48 h
AGS - 24 h
br - 24 h
AGS+br - 48 h
AGS+br+GH - AGS+br for 48 h including GH for 24 h
AGS+br+PRL - AGS+br for 24 h including PRL for 24 h.

4.3.4 Hormone replacement

Replacement of growth hormone for 24 h after a similar period of double hormone depletion partially restored milk secretion rate to 23 ml/d. This represented an increase compared with hormone-depleted animals but a decrease compared with controls at the same stage of lactation. Histologically, tissue organisation and alveolar integrity was more structured than that observed in tissue after dual hormone depletion but tissue did not appear to be as organised as control lactating tissue (Fig 4.1). On the other hand, growth hormone replacement did not decrease the extent of DNA laddering (Fig 4.2, 4.4).

With prolactin replacement, milk secretion rate recovered to 29 ml/d. Alveolar structures were more apparent after prolactin replacement suggesting this treatment produced a partial recovery of tissue organisation (Fig 4.1). Prolactin replacement reduced the extent of DNA laddering to a level similar to that detected in control tissue (Fig 4.2, 4.4).

Replacement of both prolactin and growth hormone did not restore milk secretion to control rates. After 24 h of dual hormone replacement in rats the milk secretion rate was 30 ml/d, similar to that observed with prolactin alone. However, dual hormone replacement restored tissue histology to that of day 14 lactating tissue (Fig 4.1), and DNA laddering was reduced to control levels in the tissue (Fig 4.2, 4.4).

4.4 Discussion

Litter removal in rats increased DNA laddering in mammary tissue, as it did in mice. DNA ladders of increasing intensity were detected within 24 h of litter removal and remained high in the following 24 h period. However, some mechanistic differences were detected in the process of apoptosis in mice and rats. In mice, epithelial cells were shed into the lumen during apoptosis; this was not observed during rat mammary apoptosis. Another difference between mice and rats was observed by DAPI staining, which revealed crescent-shaped nuclei in rat tissue after 24 h litter removal. Crescented nuclei, which have been reported to form prior to endonuclease activation (Walker et al., 1988) were detected in rat tissue at a time when gland DNA content had not yet decreased (D.J. Flint, personal communication), suggesting that a number of cells were poised to enter apoptosis. Crescent-shaped nuclei are also a feature of mitotic cells or may also be detected, artifactually, in tissue sections due to the plane of sectioning. However, detection of these structures in involuting rat but not mouse mammary tissue argues against artefactual apoptotic crescents and suggests that subtle differences may even exist in the apoptotic process of closely related species.

Milk secretion can be interrupted either by removal of the suckled young, whereupon milk accumulation occurs and milk synthesis ceases, or by hormonal deprivation, when milk synthesis is markedly decreased but milk removal continues due to the presence of the young. These two models provide a useful comparison of involution induced by hormonal withdrawal with that induced by milk stasis within the gland. Intense apoptosis detected in both situations highlights the multifactorial control of apoptosis. Lactation is only maintained while both milk removal and galactopoietic hormone release are maintained.

The enhanced DNA laddering after prolactin neutralisation was consistent with

other studies where prolactin has been shown to be essential for maintaining lactation and inhibiting apoptotic cell death in mammary tissue (Sheffield and Kotolski, 1992; Atwood et al., 1995). Prolactin neutralisation was also accompanied by a decreased milk yield, probably due to a reduced cell number in the gland. It is not known if this fall in cell number is a direct effect of the inhibition of the apoptotic machinery by prolactin, or if it is an indirect consequence of milk stasis. Prolactin withdrawal resulted in the production of a highly viscous lipid-rich milk which may inhibit milk removal by the pups. When pups do not or cannot obtain milk from their mother milk accumulates within the gland and autocrine feedback decreases milk secretion from these glands (Wilde et al., 1995a). In this respect, prolactin withdrawal may be likened to studies where milk composition has been altered by decreasing the α-lactalbumin gene. Mice deficient in α lactalbumin produced a viscous milk with a high lipid content and resulted in death of the pups due to an inability to remove this milk from the mother (Stacy et al., 1995). The production of a lipid-rich milk suggests that individual milk constituents can be regulated by different hormonal controls. During development the detection of ß casein after day 8 of gestation (Sympson et al, 1994) and of whey acidic protein only beyond day 14 of gestation (Pittius et al., 1988a,b; Sympson et al., 1994) suggests different milk constituents can be separately regulated. Recent work has also shown ß casein to be regulated by laminin (Streuli et al., 1995a) whereas whey acidic protein is regulated by collagen (Lin et al., 1995). Therefore although some factors, including autocrine factors, may regulate milk secretion coordinately (Wilde et al., 1995b), differential regulation of individual constituents is also likely.

The effect of growth hormone withdrawal was a slight reduction in milk yield;

conversely, one effect of growth hormone replacement was increased milk yield. Removal of either growth hormone or bromocriptine reduced milk yield to differing extents and subsequent re-instatement of growth hormone or prolactin resulted in a partial restoration of milk yield. The increased effectiveness of prolactin in inducing changes in milk yield suggests a different mechanism of action for prolactin and growth hormone. Prolactin activity primarily caused changes in the rate of apoptosis whereas growth hormone did not affect DNA laddering. The effect of growth hormone was more obvious after dual hormone withdrawal when growth hormone was replaced. In this instance, gland morphology recovered towards that observed in lactation, and milk yield increased to a level comparable with that achieved with prolactin in similar circumstances. This suggests that growth hormone does indeed influence milk yield, but may be acting at the level of cellular differentiation rather than at the level of cell death.

Growth hormone altered the extent of apoptosis only when acting in conjunction with prolactin. Apoptosis induced by prolactin withdrawal increased when growth hormone was also removed, as was the effect on milk yield. Likewise, the decrease in mammary apoptosis after prolactin replacement in bromocriptine and anti growth hormone treated rats was much greater if growth hormone was also replaced. This demonstrates a role for growth hormone throughout lactation, and suggests that growth hormone levels can alter the gland's response to prolactin. Indeed growth hormone has been demonstrated as important for maintaining milk production in the rat when circulating prolactin concentrations are low (Madon et al., 1986; Barber et al., 1992; Flint et al., 1992).

This study provided the first attempt, to our knowledge, to reverse the

progression of mammary apoptosis. Reversibility of the rate of apoptosis suggests asynchrony in the time taken after the application of an apoptotic stimuli for different epithelial cells to reach a "point of no return" from apoptotic death. Prolactin acted as a cell survival factor when replaced alone, and when added together with growth hormone. The survival mechanism due to prolactin administration is not understood, but recent experiments suggest that prolactin, unlike progesterone or glucocorticoid, does not influence mammary cell survival by a local mechanism (Feng et al., 1995). It therefore seeems most likely that prolactin acts systemically or by an indirect mode of action. Cellular response to prolactin involves a large number of cells, as can be illustrated by the increase in DNA laddering within 24 h of prolactin withdrawal and also by the reversal in apoptotic rate after 24 h of prolactin replacement.

In this chapter the effects of prolactin and growth hormone have been investigated during rodent lactation. The role of these two hormones is known to differ during the lactation cycle of rodents and ruminants. It is therefore likely that growth hormone and prolactin play different roles in altering cell susceptibility to apoptosis in rodents and ruminants. With this in mind a study of apoptosis during ruminant lactation and involution will be described in Chapter 5.

CHAPTER FIVE

APOPTOSIS IN RUMINANT LACTATION

5.1 Introduction

In the preceding chapters, cell loss during involution in mice and rats was shown to occur by apoptosis. This process was induced when milk removal ceased, for example when a litter was removed from the mother during lactation. Indeed, it appeared to be a prerequisite for mammary remodelling in preparation for the next lactation. Apoptosis occurred when the young were weaned onto solid food, progressively taking less milk from the dam. Apoptotic cell removal occurred even when the interval between each lactation cycle was reduced to a minimum by mating at *post partum* oestrus.

Some of the factors which regulate apoptosis in mammary tissue have been identified. In this project manipulation of hormonal status in the rat has been shown to result in reversibly-inducible cell loss by apoptosis, and the existence of local control mechanisms sensitive to milk stasis has been demonstrated in the mouse. It is therefore probable that both local and systemic factors are involved in the induction of apoptosis after litter removal or natural weaning in mice.

In contrast to the extensive literature on regulation of mammary cell number in rodents, the role of apoptosis in ruminant lactation is unclear. Indeed, cell loss between lactations is thought to be less important in ruminants than in rodents (Hurley et al., 1989). However, during declining lactation in goats (weeks 8 - 23) a 50 % reduction in cell number occurs (Knight and Peaker, 1984). This suggests that cell

removal may be important during declining lactation.

This study was undertaken to determine if goat mammary cell loss during declining lactation is due to apoptotic death and if so, whether it is subject to regulation by local intra-mammary mechanisms sensitive to milk removal.

5.2 Experimental protocol

5.2.1 Unilateral milk stasis

Twelve goats were milked twice daily throughout lactation. During declining lactation (week 25 - 41 of lactation) the right gland was dried off while the left gland continued to be milked twice daily. After 2 days, 1, 2 or 3 weeks of unilateral milking, tissue samples were taken from each mammary gland *post mortem.*

5.2.2.Unilateral manipulation of milking frequency

Four goats were allowed to suckle their young to establish lactation. On day 4-5 of lactation the goats were milked, individual-gland milk yield from each gland was measured and a biopsy was taken from each mammary gland (pre-treatment samples). The right gland was then switched to thrice daily milking while the left gland was milked once daily. Milk yield of each gland was recorded. After 4 weeks of this treatment a biopsy was taken from each gland (post-treatment samples).

5.3 Results

5.3.1 Unilateral milk stasis

Tissue from glands milked twice daily throughout declining lactation showed tissue morphology characteristic of secretory tissue with no evidence of involution at any stage (Fig 5.1, 5.2). Ethidium bromide staining did not detect DNA laddering in milked glands. However, [^{32}P] dCTP nick end-labelling of DNA revealed DNA laddering in milked glands which increased in intensity with duration of milk stasis in the contralateral gland (Fig 5.3).

Histological study showed tissue disorganisation in unmilked glands, which increased with duration of milk stasis (Fig 5.1, 5.2). After three weeks of unilateral milk stasis the morphology of the unmilked gland had degenerated to such an extent that alveolar organisation was no longer apparent (Fig 5.1, 5.2). DNA laddering was detected by [^{32}P] end-labelling DNA from unmilked glands after two days of treatment (Fig 5.3). Laddering in the unmilked glands increased in intensity with duration of milk stasis and was consistently of greater intensity than that observed in milked glands (Fig 5.3).

5.3.2 Effects of unilateral manipulation of milking frequency

In a second experiment goats in early lactation were unilaterally milked once and thrice daily. Average milk yield of thrice daily milked glands was consistently higher than that of contralateral once daily-milked glands (Table 5.1). The change in yield of the two glands was compared by calculation of the relative milk yield quotient (RMYQ). For each goat the RMYQ was greater than unity after 4 weeks of unilateral milking, indicating that milk yield had increased in the thrice daily milked gland compared to

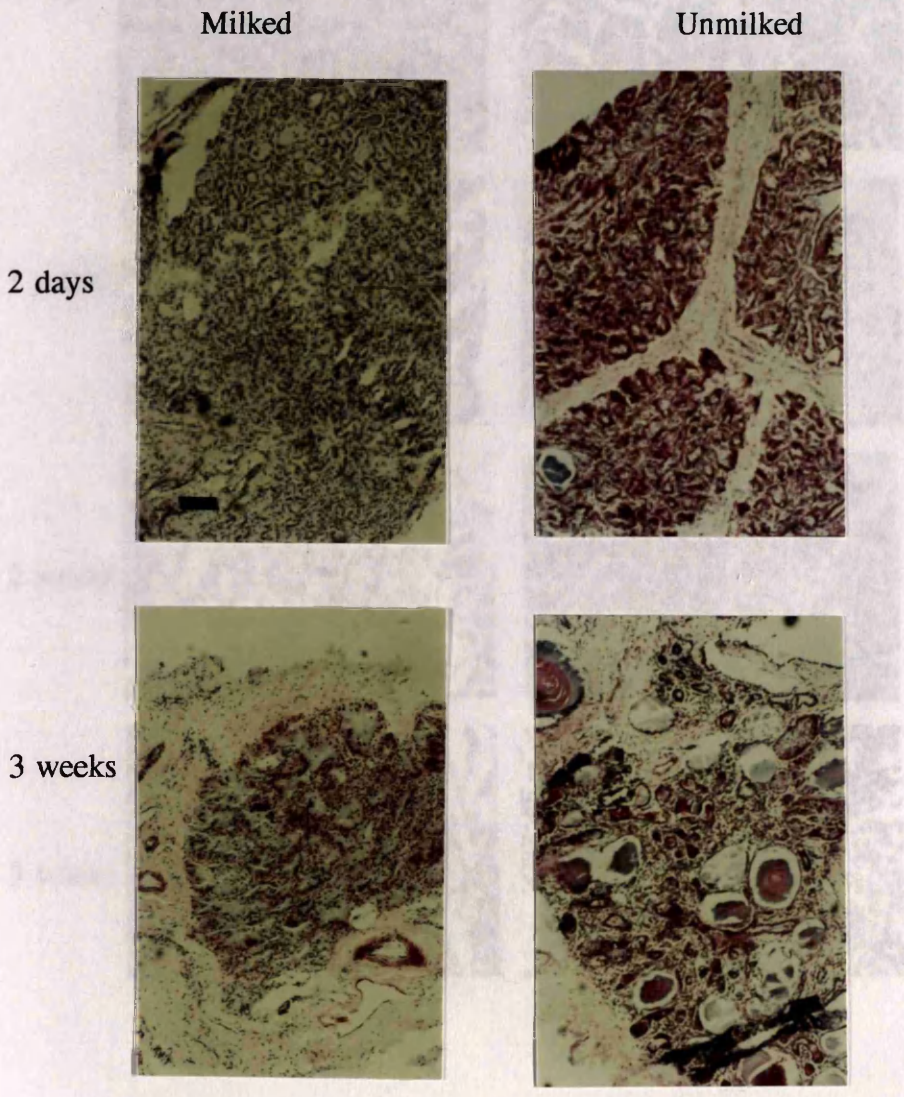


Fig. 5.1 Histology of goat mammary gland after unilateral cessation of milking . Tissue was collected *post mortem* from glands milked or unmilked for 2 days or 3 weeks during declining lactation. Sections were stained with Casson's Trichrome. Bar = 200 μm.

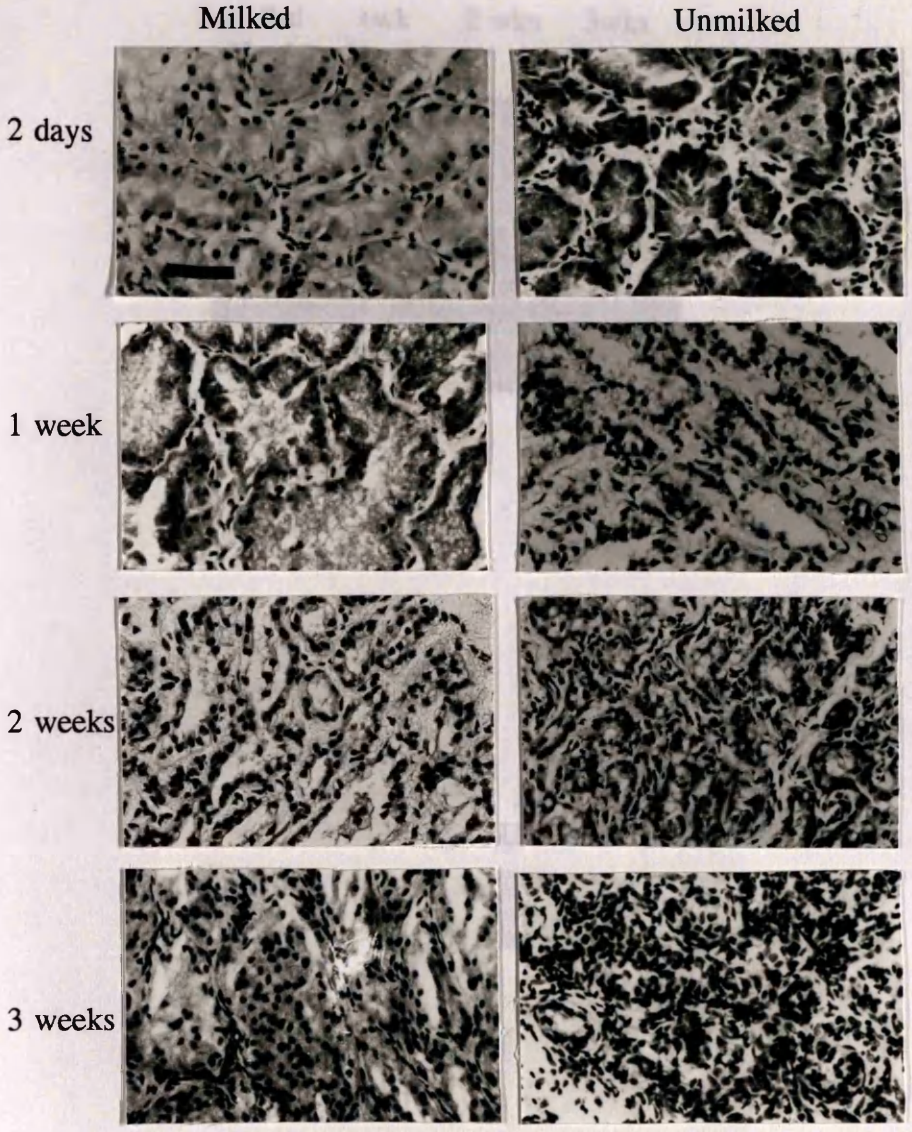


Fig. 5.2 Histology of goat mammary gland after unilateral cessation of milking. Tissue was collected *post mortem* from glands milked or unmilked for 2 days, or 1,2 or 3 weeks during declining lactation. Sections were stained with haematoxylin / eosin. Bar = 40 μm.

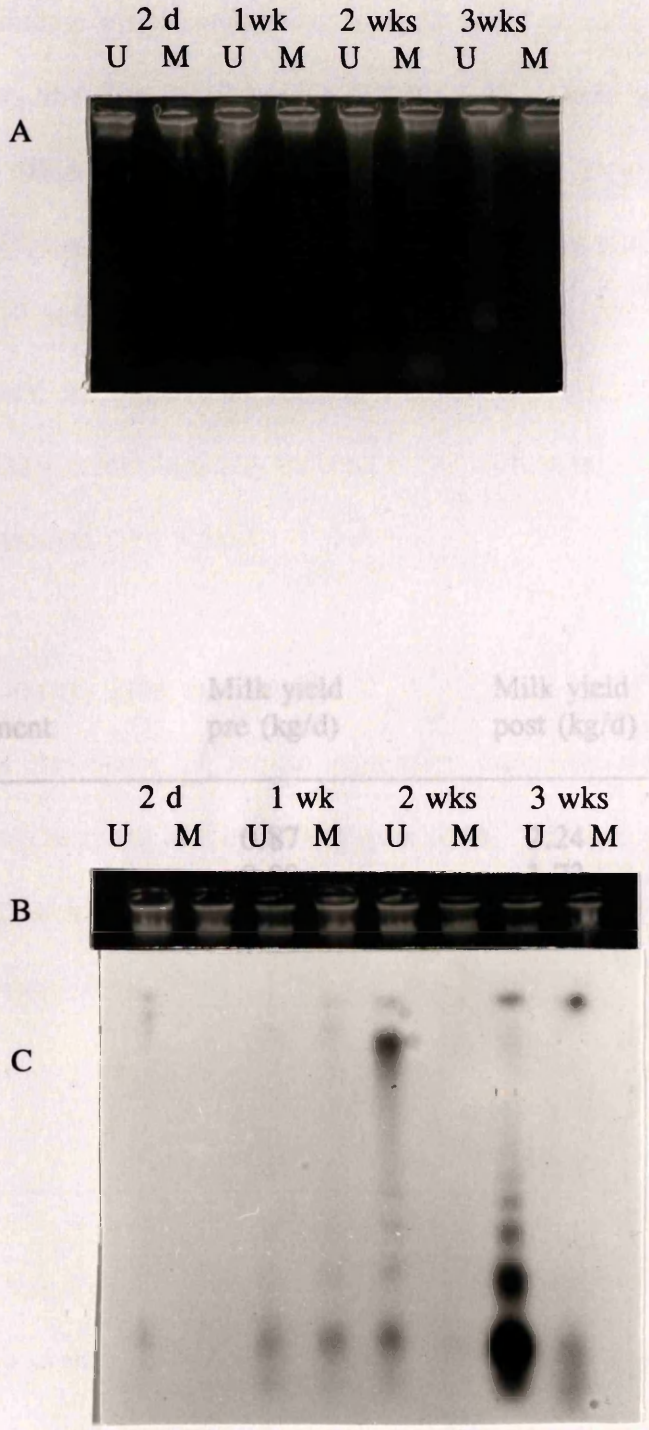


Fig. 5.3 DNA laddering induced by unilateral milking in declining lactation. A, ethidium bromide staining of electrophoresed genomic DNA. B, ethidium bromide staining of unresolved DNA to confirm equal loading of DNA. C, autoradiography of [^{32}P] d CTP end-labelled DNA. M, milked glands. U, unmilked glands.

Treatment	Milk yield pre (kg/d)	Milk yield post (kg/d)	RMYQ
3x	0.87	2.24	1.19
1x	0.80	1.73	
3x	1.23	2.53	1.52
1x	1.22	1.65	
3x	1.01	2.47	1.60
1x	0.86	1.31	
3x	1.98	3.05	1.76
1x	2.15	1.88	

Table 5.1 Milk yield after once or thrice daily milking. Milk yield was recorded for each gland after each milking. Relative milk yield quotient (RMYQ) was calculated as $(t2/c1)/(t1/c2)$, where $c1$ is the pretreatment milk yield of the control gland, $t2$ is the pretreatment milk yield of the test gland, $c2$ is the milk yield of the control gland after once daily milking had commenced and $t2$ is the milk yield of the test gland after thrice daily milking had commenced. A RMYQ value > 1 shows the test gland increased since the previous period relative to the control gland.

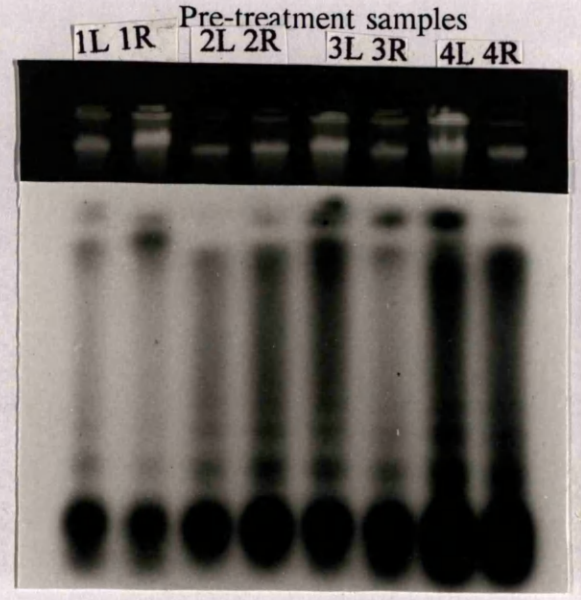
the once daily milked gland, regardless of initial yield of each gland and over and above any effects due to stage of lactation (Table 5.1). There was a marked change in the level of DNA laddering during the experimental period. Extensive DNA laddering was detected in all pre-treatment samples, and was uniformly less intense in all post-treatment samples. No differences were seen in DNA laddering of post-treatment samples, irrespective of milking frequency (Fig 5.4). Tissue from pre-treatment samples was histologically indistinguishable from once or thrice-daily milked tissue at peak lactation (Fig 5.5).

5.3.3 Goat mammary gene expression

Probes used in the study of mouse mammary apoptosis were tested for cross hybridisation with lactating and involuting goat RNA. Of these only *TIMP-2* and *bax* cross hybridised with goat RNA. Therefore, in the absence of more comprehensive information, results are not presented.

5.4 Discussion

Apoptosis was detected throughout ruminant lactation, however the extent of apoptosis varied according to the stage of lactation. Intense DNA laddering was detected in the first days of lactation, a time when cell division occurs in the mammary gland (Anderson et al., 1981; Knight and Peaker, 1984). Therefore, mammary development in early lactation may well involve mitosis and simultaneous apoptosis. The apoptosis detected during mammary development in early lactation may represent death of epithelial cells, however the removal of other cell types is a distinct possibility, since



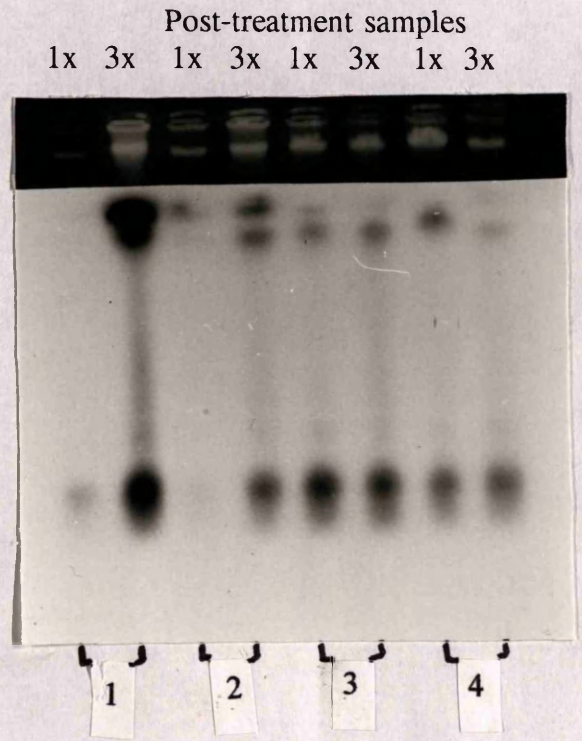
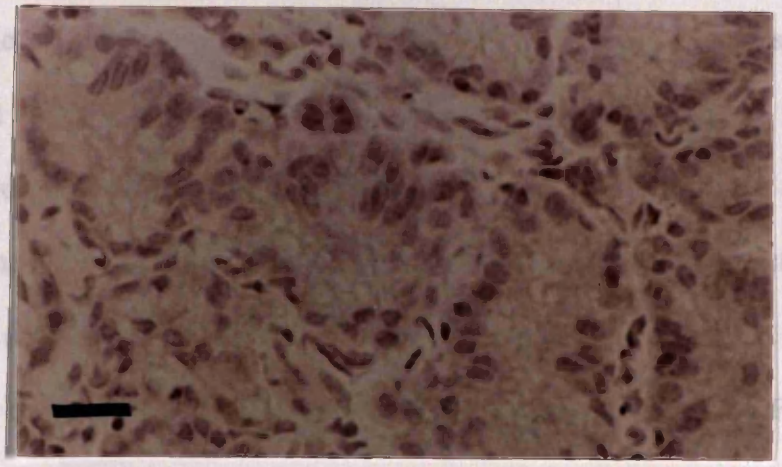


Fig. 5.4 DNA laddering before and after unilateral alteration of milking frequency. Goat mammary tissue was collected by biopsy before (Pre-treatment) and after 4 weeks of milking one gland once daily (1x) and the other gland thrice daily (3x).

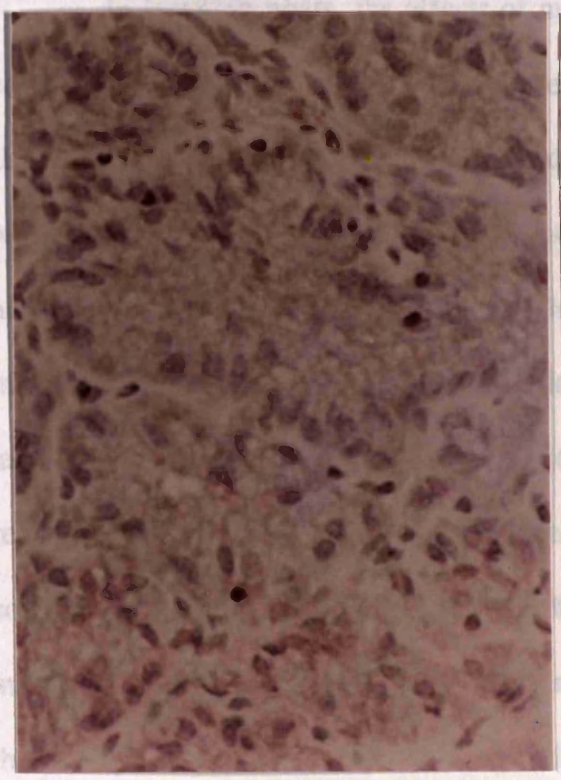
4 animals were used (1, 2, 3 and 4). L, left hand side. R, right hand side.

Pre-treatment



Post-treatment

1x



3x

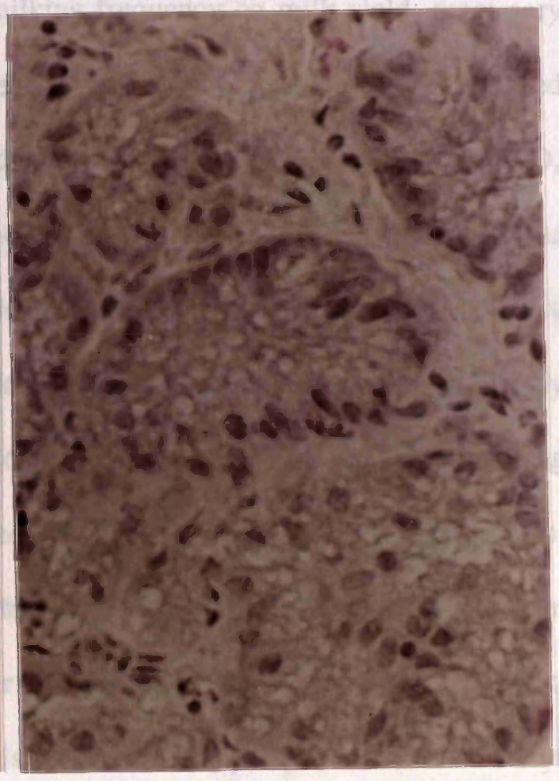


Fig. 5.5 Histology of once and thrice daily milked glands. Goat mammary tissue was collected by biopsy before (pre-treatment) and after 4 weeks of milking one gland once daily (1x) and the other gland thrice daily (3x). Sections were stained with haematoxylin / eosin. Bar = 16 μm.

in the rodent mammary gland apoptosis of epithelial and myoepithelial cells has been reported (Walker et al., 1989). During many developmental processes in other tissues, for example during the development of *C. elegans*, high mitotic indices coincided with high levels of apoptosis (Yuan and Horvitz, 1992).

Demonstration of DNA laddering, at peak lactation, albeit at a low level, suggests that cell turnover is a normal physiological process in lactating ruminant mammary tissue. Laddering was also detected in declining lactation, at a time when mammary cell number was decreasing progressively (Knight and Peaker, 1982a). Therefore, the fall in milk yield which characterises much of lactation in ruminants may well be due to cell removal through programmed cell death.

In lactating goats, the effects of milking frequency on milk yield, mammary cell secretory activity and mammary cell number have been well documented (Henderson et al., 1983; 1985; Knight and Wilde, 1987; Wilde et al., 1989; 1991), whereas the importance of cell death and the role of apoptosis is unknown. There was no difference in the extent of DNA laddering induced under once or thrice daily milking in early lactation: instead changes in DNA laddering in the two glands in early lactation reflect differences due to stage of lactation rather than effect of milking regime. In both cases, laddering decreased dramatically after 4 weeks of treatment compared to a few days of lactation. This suggests that short term alterations in milking frequency do not alter cell number or apoptosis in the tissue. Nevertheless, the milk-yield of thrice daily milked glands was consistently higher than once daily-milked glands. This suggests that short term alterations of milking frequency do not lead to changes in cell number, but that the altered milk yield is achieved through changes in cell differentiation. Indeed, markers of cell differentiation were elevated

in thrice daily milked glands as compared to once daily milked glands (J. Brown, personal communication) and milk protein mRNA abundance was higher in thrice daily milked glands than in once daily milked glands (J. Bryson, personal communication). Therefore this study supports previous data which show short term milking responses are due to cell differentiation responses. Short term once daily milking resulted in decreased milk yield due to reduced differentiation of secretory cells (Wilde et al., 1989; Wilde and Knight, 1990). Conversely the increase in milk yield due to short term thrice daily milking has been found to be due to increased secretory cell differentiation (Akers and Heald, 1978; Wilde et al., 1987b).

To produce changes in the level of mammary apoptosis which might account for the effect of milking frequency on cell number observed in earlier studies the milking stimuli must be of long duration or more intense nature. When frequent milking was sustained for several months gland size increased, suggesting either growth or reduced regression of the thrice daily milked gland (Wilde et al., 1987b). An increase in DNA synthesis due to long term thrice daily milking (26% higher after 37 weeks of treatment) suggested a growth response as opposed to merely a reduction in the rate of involution (Wilde et al., 1987b). However, the fact that proliferation was increased does not rule out the possibility of reduced cell death.

The stimulus of milk stasis was so intense that an increase in apoptosis was initiated within one week. DNA laddering increased with duration of milk stasis, indicating that apoptosis was increased as involution progressed in goat mammary tissue. Therefore, as in rodents, apoptosis plays a central role in cell removal after milking ceases. The mechanism of local control of apoptosis, demonstrated during unilateral cessation of milking in goats, is not yet understood. However, it is likey that

a similar mechanism operates in rodents since local control of apoptosis has been demonstrated after unilateral teat sealing in mice (Chapter 3). Local control in mammary tissue may be due to the physical presence of milk in the gland or may be chemical in nature. A constituent responsible for autocrine control has been purified and termed FIL (feedback inhibitor of lactation; Wilde et al., 1995a). The role this factor plays influencing apoptotic rates in the mammary gland has yet to be investigated. In goats, evidence for increased apoptosis was also apparent in milked glands with increased duration of cessation of milking in the contralateral gland, albeit to a lesser degree than in non-milked glands. The same was also true during unilateral teat sealing in mice, where apoptosis was always more prevalent in sealed glands but apoptosis also increased with duration of teat sealing in sealed and unsealed glands. These obsevations in ruminants and rodents suggest a role for systemic influence during local control of apoptosis.

The detection of DNA laddering in early lactation, at peak lactation, in declining lactation and during gland involution suggests that apoptosis is part of the normal repertoire of goat mammary tissue. It had been suggested that involution and cessation of milk production in the dairy cow does not involve massive cell death (Hurley et al., 1989). However, the widespread incidence of apoptosis in ruminant as in rodent mammary gland during involution highlights similarities in the importance of apoptosis in the two species despite selection of ruminants for milk production.

CHAPTER 6

MECHANISM OF MAMMARY APOPTOSIS

6.1 Introduction

Apoptosis was observed consistently and on a significant scale at the end of lactation in mice, rats and goats. These studies demonstrated that apoptosis was regulated by both the endocrine system and by locally acting factors operating within the gland. The two mechanisms, either alone or in combination induced apoptosis after teat sealing, litter removal or during natural weaning in rodents. Local and endocrine mechanisms also combined to regulate apoptosis in ruminants, as demonstrated by detection of cell death in milked and unmilked glands after unilateral cessation of milking.

A mammary epithelial cell culture system was required to investigate the regulatory mechanisms indicated by the *in vivo* experiments. The use of recently developed cell culture techniques has enabled culture of epithelial cells upon a layer of reconstituted basement membrane extracted from Engelbreth-Holm-Swarm mouse tumour (EHS matrix; Barcellos-Hoff et al., 1989, Aggeler et al., 1991). The EHS matrix provides a scaffolding of extracellular constituents which mimics the basement membrane that surrounds mammary epithelial cells *in vivo*. This EHS matrix contains laminin, collagen, fibronectin and a variety of uncharacterised extracellular components which can alter the behaviour of adjacent cells. Use of the EHS matrix during mammary epithelial cell culture enabled study of differentiated rather than proliferating cells and the mechanisms of vectorial milk secretion (Barcellos-Hoff et al., 1989; Aggeler et al., 1991; Talhouk et al., 1992; Streuli et al., 1993; Blatchford et al.,

1995). The interactions between mammary epithelial cells and the extracellular matrix were examined in this system to determine if it offered a means of studying the mechanism and regulation of mammary apoptosis.

6.2 Experimental protocol

In the previous chapters apoptosis has been studied by inducing cell death in well differentiated cells *in vivo*. To sucessfully study apoptosis in a cell culture system the mammary epithelial cells were plated on top of a layer of EHS matrix as this is the best way to obtain well differentiated cells in culture. The epithelial cells on EHS differentiated to form alveolar like structures with a central lumen, a situation which resembles mammary cell assembly *in vivo*. Therefore this cell culture system combined the study of relationships between mammary structure and function with advantages of cell accessability, to enable a more complete dissection of the regulation of apoptosis than is possible from *in vivo* studies.

The incidence of DNA laddering was studied throughout the period of cell culture, but a peak of endonuclease activity at the third day of culture focussed attention on the role of apoptosis during mammosphere formation. The mechanism of apoptosis during the initial days of culture was investigated by laminin staining of mammosphere structures and by determining levels of insulin growth factor binding proteins (IGFBP's) in conditioned media.

6.3 Results

In the first few days of culture cells plated as clumps of cells attatched to the matrix and then gradually became enshrouded in matrix which was pulled from the tissue culture plastic (Fig 6.1). Initially these structures contained a core of epithelial cells, however after several days in culture the clumps of cells became hollow consisting of a complete sphere of cells enclosing one or more large central spaces or lumina (Fig 6.2). These structures are termed mammospheres. Vectorial ß casein secretion was observed after day 3 of culture (Fig 6.3). Whey acidic protein, which is secreted by differentiated mammary epithelial cells, was detected after day 3 of culture, and was also secreted vectorially in the forming luminal space of the mammospheres (Fig 6.4). Not all milk proteins exhibited vectorial secretion after mammosphere formation. For example, transferrin was released from basal and apical cell surfaces before and after lumen formation (Fig 6.5).

DNA laddering was detected throughout the course of culture (Fig 6.6) and was consistently higher throughout the culture period than during lactation in mice (data not shown). On the third day of culture laddering was particularly intense (Fig 6.6). This transient increase in DNA laddering coincided with lumen formation. DNA laddering also coincided with detection of apoptotic bodies which were observed at day 3 and 4 of culture (Fig 6.7). Apoptotic bodies were localised to central areas of the mammospheres (Fig 6.7), whereas nuclear chromatin appeared intact in peripherally located nuclei.

Prior to lumen formation, immunocytochemical staining revealed peripheral cells (Fig 6.8) in contact with laminin (the major component of the extracellular matrix), while cells in the centre of the clump were not associated with laminin (Fig

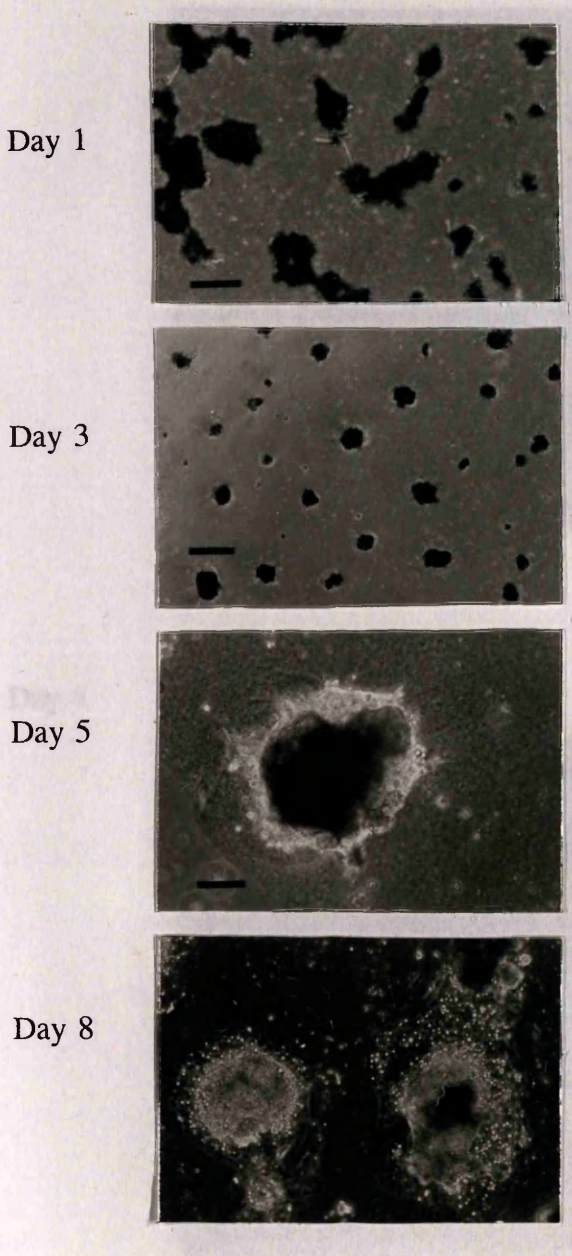
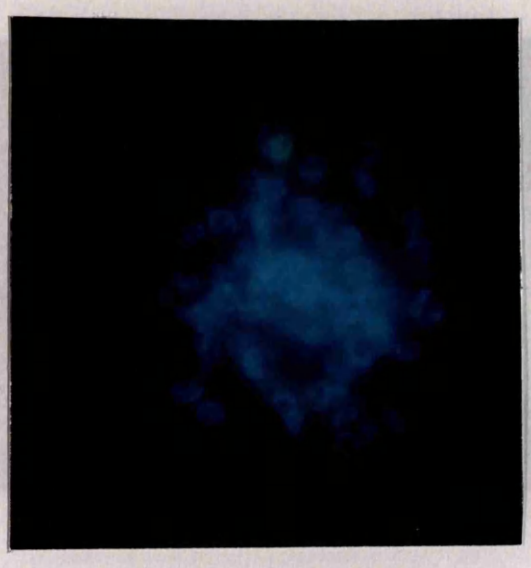


Figure 6.1 Appearance of mouse mammary cell clumps plated on extracellular matrix. Cell clumps were plated on extracellular matrix for 10 days. Cells were photographed on day 1,3,5 and 8, just prior to a daily media change. Days 1 and 3 bar = 200 μm. Days 5 and 8 bar = 30 μm.

Day 2



Day 5

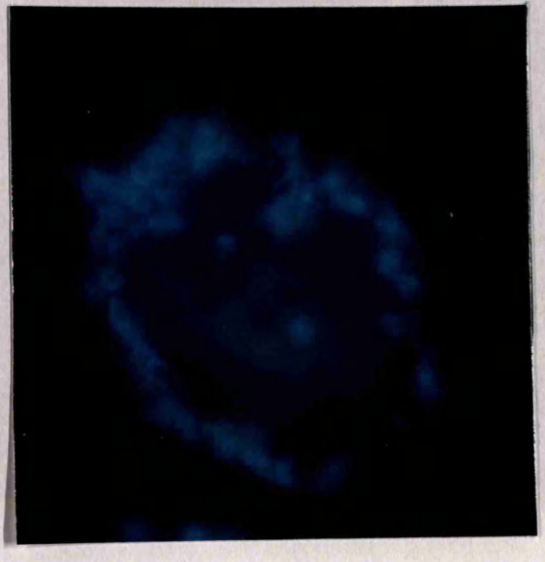


Figure 6.2 Formation of lumina in mouse mammary cell clumps. Cells were fixed in 4% (w/v) paraformaldehyde on days 2 and 5, harvested by cell scraping and embedded in OCT for frozen sectioning. 5μm sections were visualised by phase microscopy to visualise lumina formation. Bar = 50 μm.

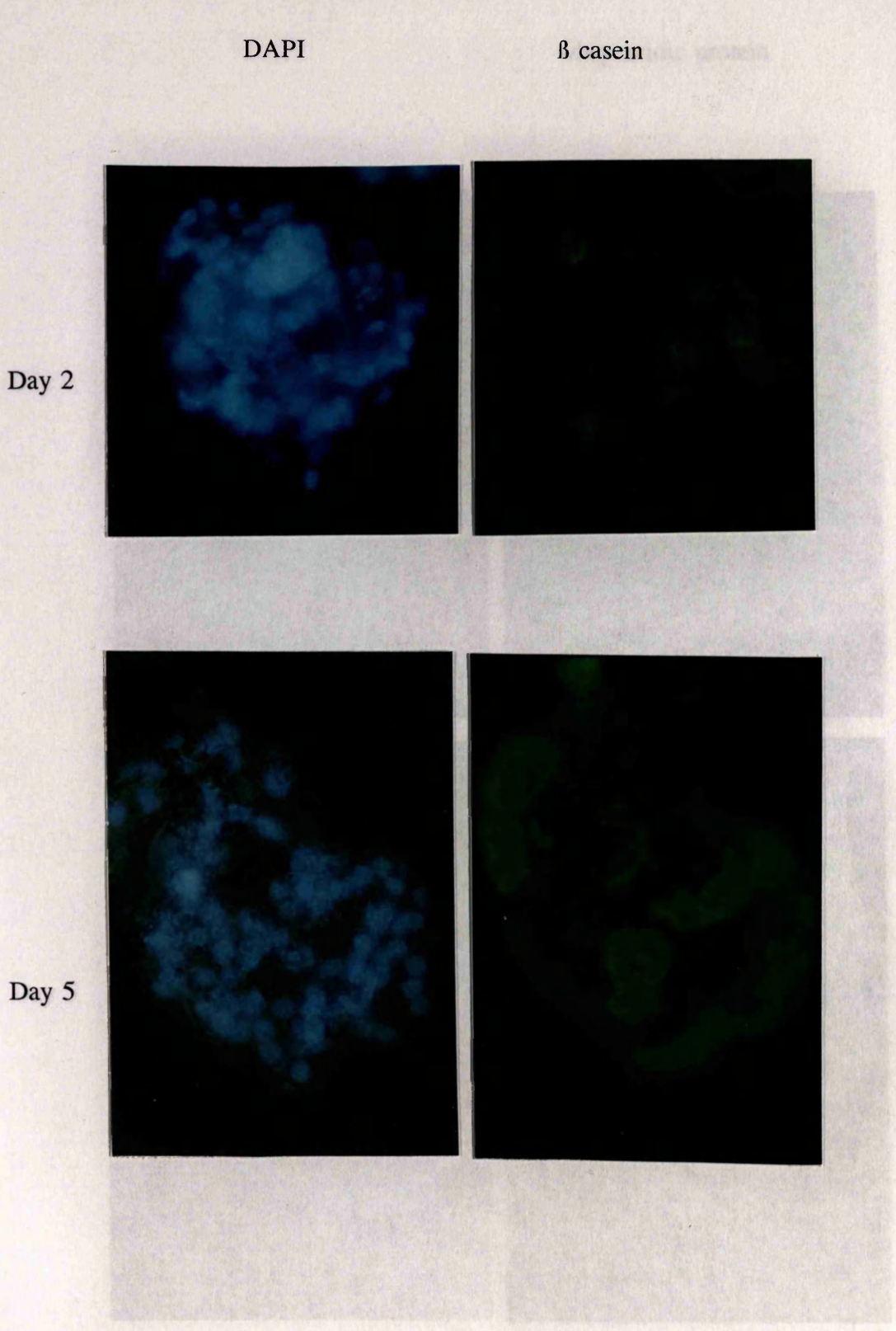


Figure 6.3 Secretion of milk components by the mouse mammospheres. 5μm frozen sections of cell clumps at days 2 and 5 of culture were stained with anti-mouse ß casein antibody, counterstained with DAPI and visualised by fluorescent secondary reagents. Sections were visualised by fluorescent microscopy. Bar = 50 μm.

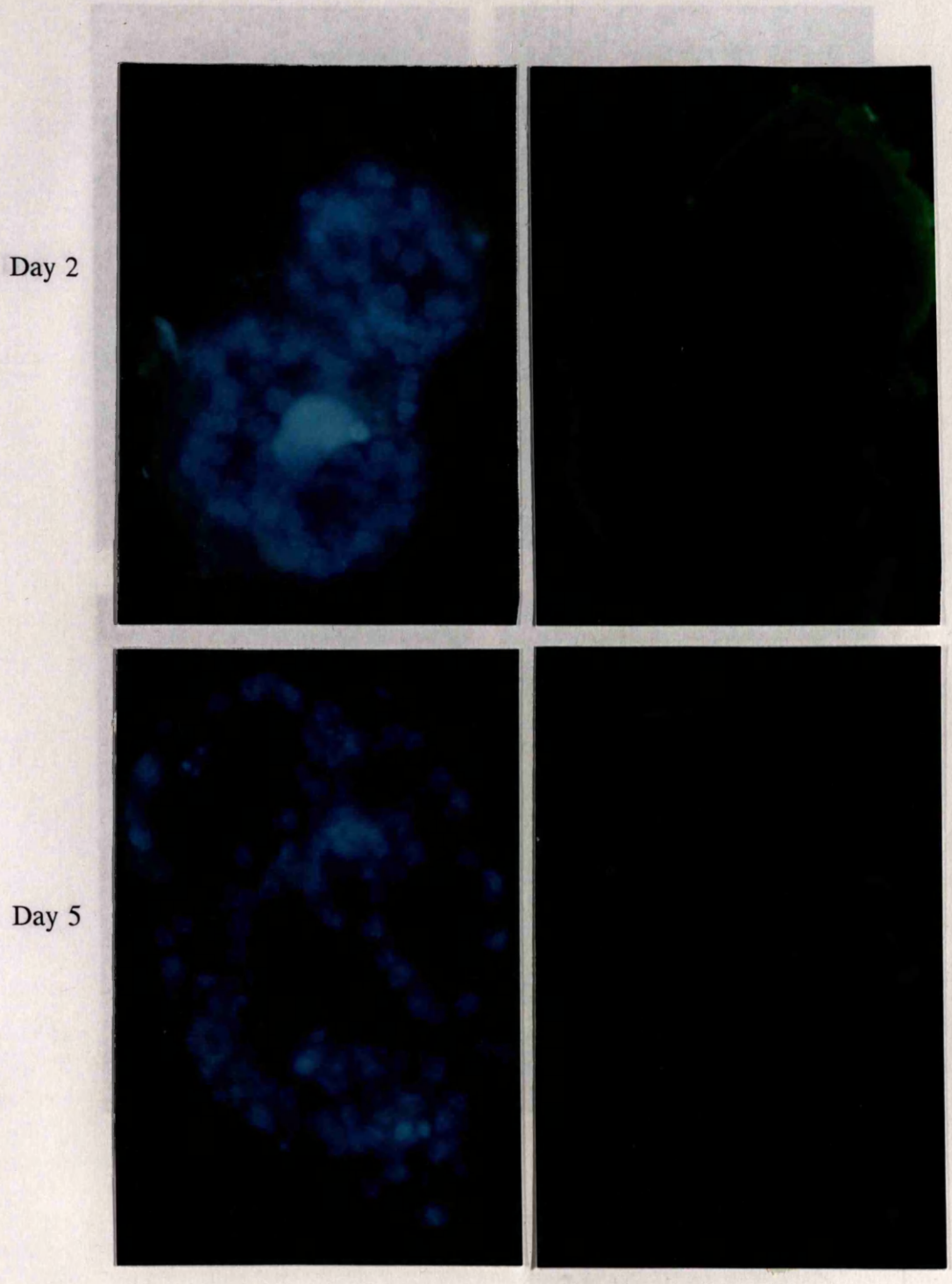


Figure 6.4 Secretion of milk components by the mouse mammospheres. 5μm frozen sections of cell clumps at days 2 and 5 of culture were stained with anti-mouse whey acidic protein antibody, counterstained with DAPI and visualised by fluorescent secondary reagents. Sections were visualised by fluorescent microscopy. Bar = 50 μm.

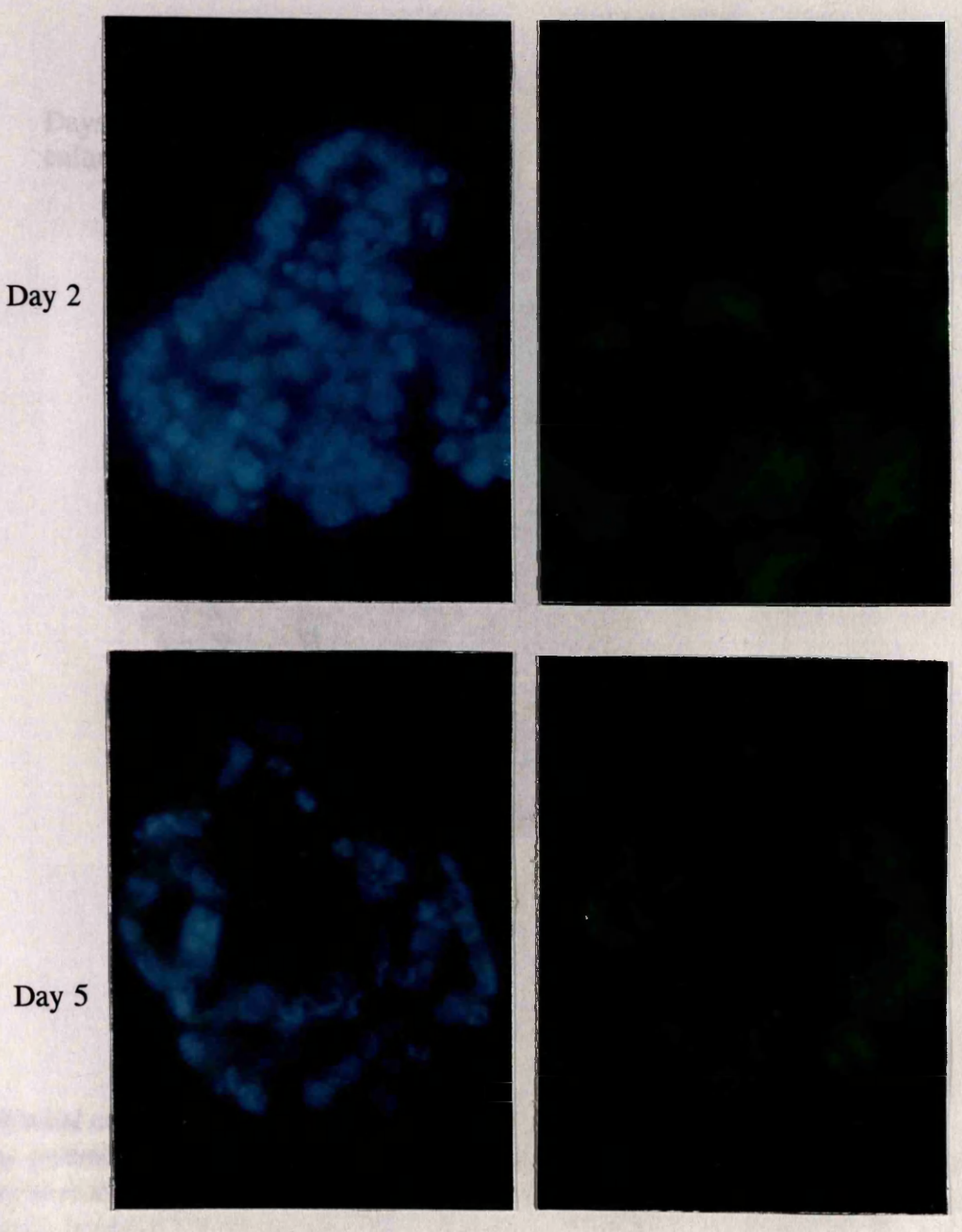
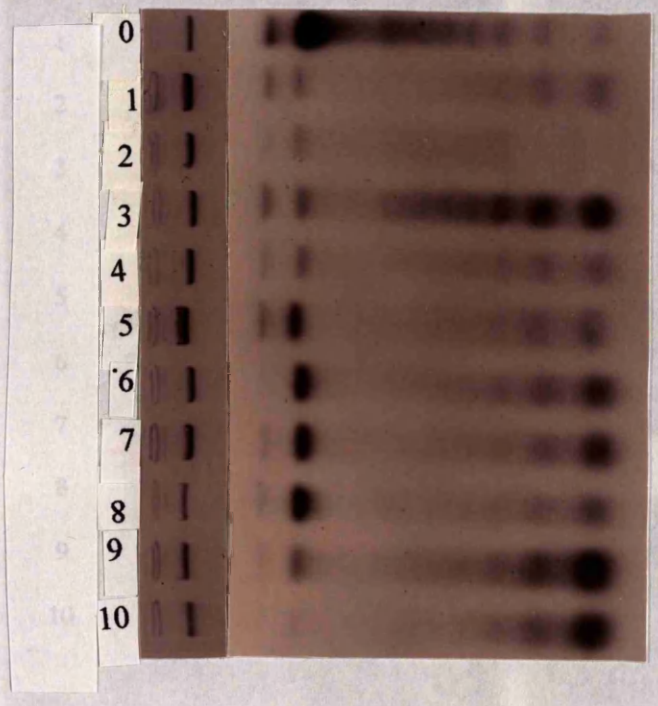


Figure 6.5 Secretion of milk components by the mouse mammospheres. 5μm frozen sections of cell clumps at days 2 and 5 of culture were stained with anti-mouse transferrin antibody, counterstained with DAPI and visualised by fluorescent secondary reagents. Sections were visualised by fluorescent microscopy. Bar = 50 μm.



These epithelial cell samples are unlikely to contain any extracellular matrix, as the cell harvesting method (as described in 2.3.2) involved use of dispase, an enzyme that was used to strip the epithelial cells away from the extracellular matrix, further, cells were also washed in culture media between harvesting and DNA extraction procedures (as described in 2.3.2) and therefore any laddering due to extracellular matrix ought to be eliminated.

Figure 6.6 DNA laddering in mouse mammary epithelial cells on EHS matrix. Cells were harvested daily with dispase, genomic DNA was isolated using the Puregene DNA kit, and DNA was nick-end labelled with [^{32}P] dCTP prior to electrophoresis and autoradiography. A, ethidium bromide staining of unresolved DNA in sample wells. B, DNA fragmentation visualised by autoradiography. This experiment was repeated three times.

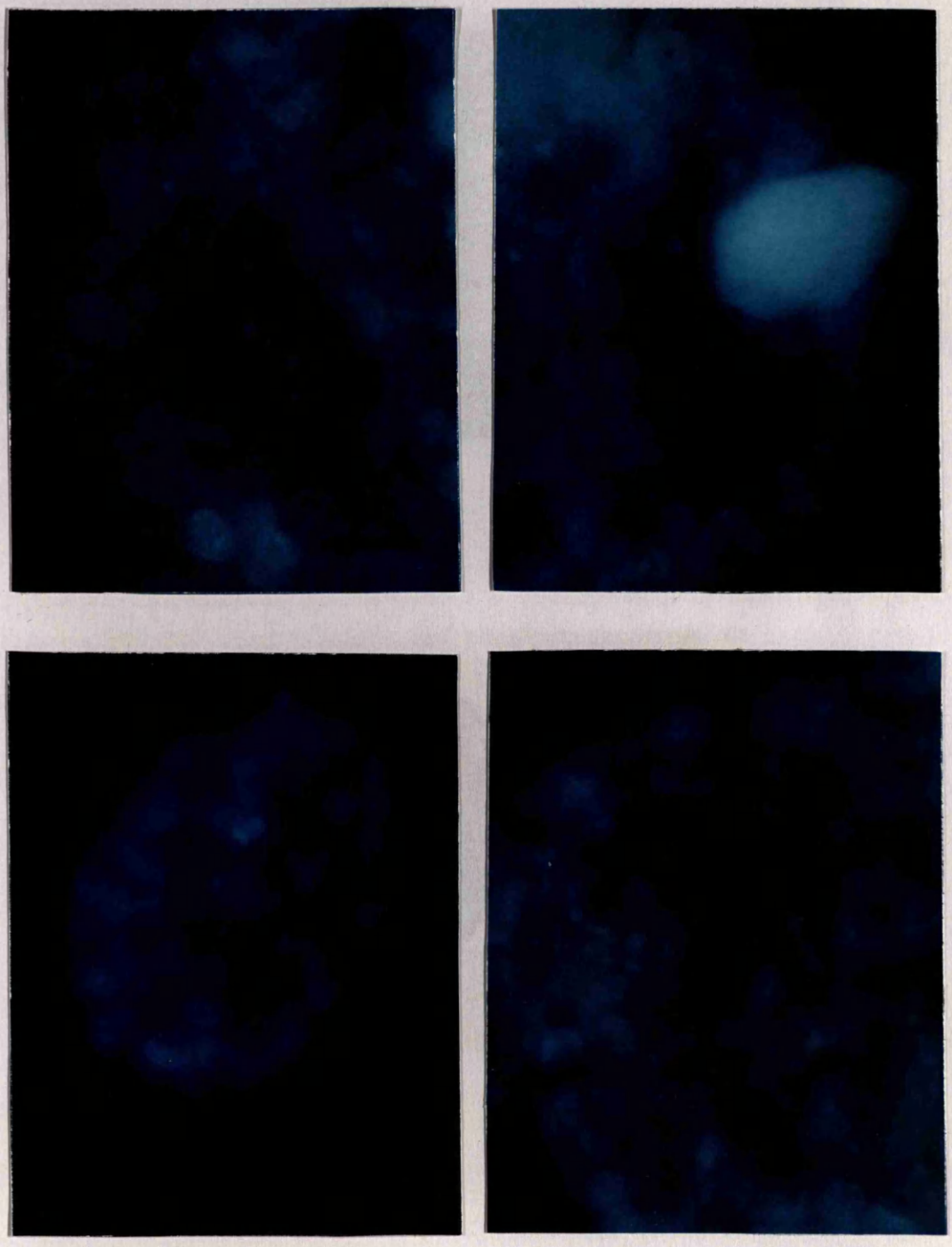
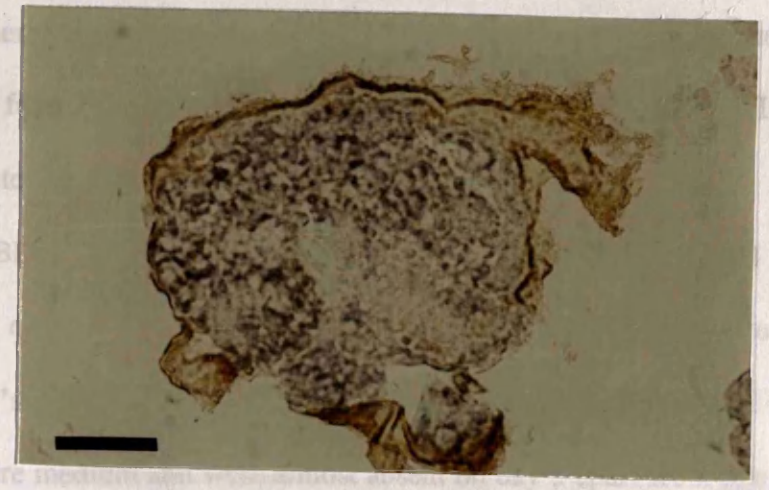


Figure 6.7 Apoptotic bodies within the mouse mammary cell clumps at day 3 or 4 of culture. 5μm sections of mouse mammospheres stained with DAPI and visualised by fluorescent microscopy. Bar = 5 μm.

Day 2



Day 5

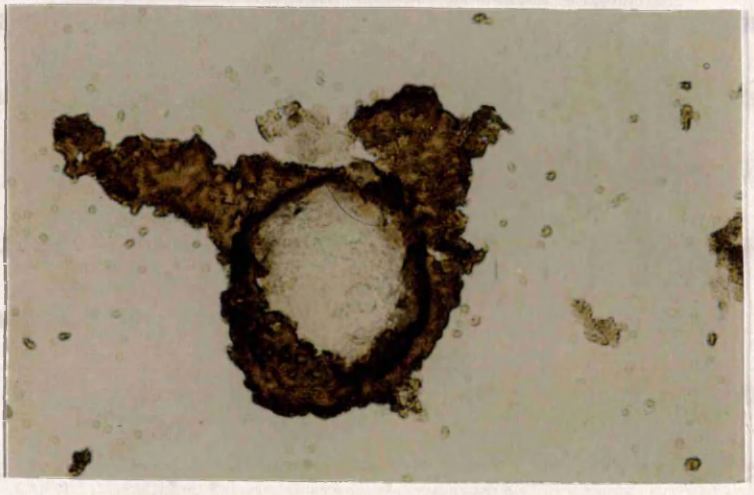


Figure 6.8 Laminin staining of mouse cell clumps. 5μm sections of cell clumps at days 2 and 5 of culture were stained for presence of laminin and staining was visualised by diaminobenzidine. Bar = 50 μm.

6.8). After lumina had formed, laminin was detected as a thicker layer on the periphery of the mammosphere and was absent from the mammosphere interior (Fig 6.8). Whether this thickening is due to laminin secretion by the outer cells or to appropriation from the matrix is unclear. Expression of stromelysin or TIMP-2 mRNA was not detected throughout the first five days of culture.

IGFBP's were detected in conditioned media, by ligand blotting, in progressively diminishing amounts throughout the first 3 days of culture (Fig 6.9). These IGFBP's were present on day 1, were detected in greatly reduced concentration in day 2 culture medium and were almost absent on day 3 apart from one IGFBP. This was probably due to residual IGFBP's contained in serum used during the first day of culture. The IGFBP which remained detectable in conditioned media on the third day of culture was of molecular weight 30kDa (D.Flint, personal communication), suggesting that the protein was IGFBP 4 or IGFBP 5. Northern analysis, using a rat cDNA probe, was unable to confirm elevated levels of IGFBP 4 or IGFBP 5 mRNA on day 3 of culture. This may have been due to lack of cross hybridisation of the rat cDNA with mouse RNA.

Milk secretion by these mammosphere structures has been shown to occur vectorially and basolaterally by two pathways, one a calcium independent constitutive pathway, the other a regulated pathway stimulated by elevation of intracellular calcium (Blatchford et al., 1993). Of all the protein secreted by the mammospheres 41.3 + 7% has been shown to be secreted vectorially (Blatchford et al., 1995), this value was within the range reported by other laboratories (Barcellos-Hoff et al., 1989; Seely and Aggeler, 1991). Vectorial milk secretion was not just a feature of day 5 mammospheres as even at day 8 and 9 of culture the mammospheres were still

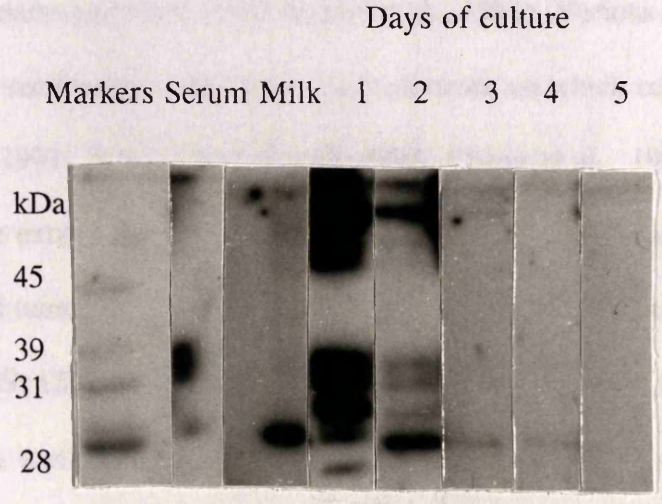


Figure 6.9 IGFBP levels in conditioned media from mouse mammary cells cultured on EHS. IGFBP's were detected by ligand blotting of conditioned media collected on days 1-5 of culture. The ligand blot was performed by Miss E. Tonner.

secreting milk proteins in a vectorial manner. However, at this stage of culture there was evidence of cell proliferation in the area surrounding the mammosphere which had been depleted of extracellular matrix (Fig 6.1). A slight increase in DNA laddering was observed at this stage of culture (Fig 6.6).

6.4 Discussion

The extracellular matrix is recognised to be a vital component of the cellular environment (Adams and Watt, 1990; Streuli et al., 1991). Various extracellular matrix components are secreted by cells to form a framework on which cells can be anchored (Streulli et al., 1991; Howlett and Bissell, 1993; Chang at al., 1995, Tremble et al., 1995). Thus the extracellular matrix provides spatial orientation and stability for cell organisation and tissue development (Kleinman et al., 1986; Li et al., 1987; Barcellos-Hoff et al., 1989; Chen et al., 1989; Aggeler et al., 1991). The extracellular matrix is not merely a scaffolding, it also takes part in the regulation of cell growth and differentiation (Adams and Watt, 1990; Streuli and Bissell, 1990; Bissell and Barcellos-Hoff, 1991).

The structure and function of normal mammary epithelia is dependent upon signals from the mammary extracellular matrix (Li et al., 1987; Schmidhauser at al., 1990; Aggeler at al., 1991; Seely and Aggeler, 1991; Streuli et al., 1991). Mammary differentiation is controlled through co-operation between extracellular components and lactogenic hormones (Topper and Freeman, 1980, Schmidhauser et al., 1990; Talhouk et al., 1992, Vollmer at al., 1993; Politis et al., 1995). The laminin-rich basement membrane, along with lactogenic hormones, has been shown to activate transcription

of milk proteins including ß casein (Li et al., 1987; Streuli et al., 1991, 1995a) and whey acidic protein (Chen and Bissell, 1989; Lin et al., 1995). Experiments in this chapter show that epithelial cell differentiation commenced during formation of the lumina, at days 3-5 of culture, a time at which vectorial milk secretion commenced.

The formation of lumina had previously been assumed to be due to cell movement of central cells towards the periphery of the cell clump. However an increase in DNA laddering at the time of lumen formation suggested the involvement of apoptosis. The presence of apoptotic bodies in the mammospheres confirmed that increased apoptosis was taking place during lumen formation. These apoptotic bodies were located in the centre of the mammospheres suggesting that luminal spaces were created by cell death rather than cell movement. In short, cell localisation appeared to determine cell fate.

The mechanisms of selective cell survival remain unclear. One candidate cell survival factor is the EHS component, laminin. Laminin is recognised by α1/ß1 and α6/ß1 integrin receptors (Hall et al 1990), which have been demonstrated as inducers of differentiation and ß casein production (Streuli et al., 1991). More recently laminin, through a specific site in its globular domain has been shown to interact functionally with mammary epithelial cells to activate ß casein gene transcription (Streuli et al., 1995a) and apoptosis has been induced on addition of ß1 integrin antibody to culture medium of a mammary cell line (Boudreau et al., 1995). This evidence suggests that extracellular laminin acts through integrin receptors to influence intracellular molecules and prevent apoptosis in mammary cells.

In the mammosphere system, cell contact with laminin seemed to be a pre-requisite for survival. The antibody used for detection of laminin production by mouse

mammary epithelial cells also inevitably detected laminin contained in extracellular matrix extracted from the mouse EHS tumour. This staining confirmed peripheral cell contact with matrix laminin during the early stages of culture and a relative absence of laminin in the core of the mammosphere. Therefore, laminin seemed to be in contact only with peripheral cells, and it was these cells which survived to form mammospheres. Cells within the clump did not sequester laminin and it was these cells which died, resulting in a hollow sphere of cells. Later in culture, after day 5, the laminin layer around the periphery of the mammosphere appeared even more densely stained than the surrounding matrix, suggesting that once the mammary epithelial cells have differentiated they may secrete laminin basolaterally (Streuli and Bissell, 1990; Howlett and Bissell, 1993).

Loss of contact with the basement membrane can induce changes in differentiation in some cell systems for example keratinocyte terminal differentiation is triggered by loss of extracellular matrix (Adams and Watt, 1990) and in other systems loss of basement membrane contact can induce death, for example survival of differentiated hepatocytes is dependent upon extracellular matrix - cell contact (Schuetz and Schuetz, 1993), demonstrating a causal link between membrane contact and survival. Therefore the proposed role for contact with laminin as a cell survival factor is not unique to mammary tissue.

Previous investigators of apoptosis during gland involution have correlated increased protease activity due to alterations in the protease : protease inhibitor ratio, with advancing apoptosis (Strange et al., 1992, Talhouk et al., 1991). During involution proteases destroy the extracellular matrix and this protease activity produces folds and convolutions in the extracellular matrix (Walker et al., 1989). This activity

breaks the contact between epithelial cells and the basement membrane, and this is thought to induce apoptosis in the cells which lose basement membrane contact (Strange et al., 1992; Talhouk et al., 1991). In culture however we did not detect expression of stromelysin (a major protease induced during mammary involution: Strange et al., 1992) during the period of lumen formation when apoptosis was increased. This suggests that loss of basement membrane contact, which induces apoptosis, may in these circumstances be due to mechanisms other than protease -induced breakdown of the basement membrane. Apoptosis was also detected at peak lactation *in vivo,* at a time when proteases were reported not to be expressed (Strange et al., 1992). It is therefore possible that apoptotic cells at peak lactation may, by some unknown mechanism, lose basement membrane contact by a protease- independent pathway, and undergo apoptosis.

IGF-1 is a potent mitogen for mammary tissue (Ruan et al., 1992; Deeks et al., 1988) and has an anti-apoptotic effect in other cell types (Rodriguez-Tarduchy et al., 1992, Drago et al., 1991; Sell et al., 1995). High concentrations of an IGF binding protein have been demonstrated in milk during involution of rat mammary gland (Tonner et al., 1995) and this IGFBP has been recently identified as IGFBP 5 by Northern analysis (M. Travers, personal communication). Western blotting has revealed the IGFBP to have an apparent Mr of approximately 30kDa, similar to that of IGFBP 5 (D. Flint, personal communication). Tenniswood and colleagues (1995) have also recently identified a presently uncharacterised IGFBP, which is elevated during mammary involution (M. Tenniswood et al., personal communication). Earlier studies by this same group detected elevated RSG 8 in prostate after androgen ablation, and demonstrated that RSG 8 codes IGFBP 5 (Guenette et al., 1994). IGFBP 5 binds

IGF-1 with high affinity and therefore sequesters IGF-1 in the extracellular matrix and attenuates the interaction between IGF-1 and the IGF-1 receptor, and induces apoptosis (Guenette and Tenniswood, 1995). Therefore in addition to regulation of apoptosis by the extracellular matrix, changes mediated through IGF-1 may be important.

Apoptosis has also been reported to occur in whole organ mammary culture upon withdrawl of hormones from the culture media (Atwood et al., 1995). This may prove to be a suitable model for studying apoptotic changes during involution of the gland at the end of lactation. However whole organ *in vitro* studies have the disadvantage that they contain both epithelial and stromal elements and so epithelial cell specific effects can not be investigated and often the tissue must be primed with hormones prior to removal form the animal, a factor which may influence many studies. Levels of apoptosis have been reported to be higher in whole organ cultures than in fully functional mammary tissue, suggesting that cell death pathways in organ cultures may be under additional pressures not experienced in lactating glands. The mammosphere cell culture system ensures that the structural and functional similarity with cells *in vivo* combined with advantages of cell accessability. The demonstration of apoptosis in this system (Boudreau et al., 1995) suggests this is a suitable system for study of mammary apoptosis. The increase in apoptosis beyond day 8 in this system may provide an *in vitro* model for the study of mammary apoptosis induced during involution at the end of lactation. This study has determined the importance of laminin in maintainance of cell integrity and the possible regulation of mammary apoptosis by an IGFBP, probably IFGBP, 5 *in vitro*. It is likely that laminin is responsible for cell survival *in vivo* and preliminary experiments suggest IFGFBP 5 may play a role during mammary apoptosis *in vivo*. The effects of manipulating levels of laminin and IGFBP

5 *in vivo*, and investigation of the influence of these extracellular signals upon systemic and local regulatory mechanisms during mammary apoptosis, will clarify the roles and possible interactions of laminin and IGFBP 5 during mammary development and function.

CHAPTER SEVEN

SUMMARY AND DISCUSSION

Mammary cell number during lactation depends upon the extent of proliferation and cell death in the gland. A dynamic balance between the two determines mammary cell number and this is a primary determinant of milk yield. Until recently changes in cell number were only considered in the context of proliferative changes (Knight and Peaker 1982a, Knight et al., 1984). The realisation that cell death, by apoptosis, could be a regulated process has occurred with the advent of techniques which can monitor the extent of DNA fragmentation.

This study has increased knowledge of the physiological role of apoptosis in mammary tissue. Perhaps most importantly this work has demonstrated that apoptosis is not reserved for gland involution, that it occurs, albeit to a lesser degree throughout lactation (Chapter 3). However, apoptosis occurs to a comparable extent in concurrently-pregnant mice and in non-pregnant counterparts (Chapter3) and mammary re-development and involution were shown to occur simultaneously (Chapter 3). Further, the project has demonstrated that mammary apoptosis is controlled both systemically and locally within the tissue (Chapters 3, 4 and 5).

The importance of apoptosis throughout a ruminant lactation cycle (Chapter 5) suggests that manipulation of apoptotic pathways may provide a means of altering ruminant cell number during lactation. One of the milking studies in Chapter 5

commenced at day 3 of lactation, a time which was subsequently found to be associated with a high number of apoptotic deaths. The reason for the high level of apoptosis in early lactation is unknown. It is possible that a reduction in apoptosis at this time may result in an increased cell number throughout lactation. Changes in milking frequency during early lactation have been shown to result in altered milk yield (Henderson et al., 1983, 1985; Knight and Wilde, 1987; Wilde et al., 1989; 1991) due to short term differentiative changes (Wilde et al., 1987b, Wilde and Knight 1990). Short term alterations in the extent of apoptosis and cell number may be achieved by immediate introduction of milking regimes upon establishment of lactation rather than at day 3 of lactation, a time by which extensive cell removal possibly could have occurred. Although at present it is unclear if at this stage dying cells are epithelial or not.

Previous studies also indicated that longer term alterations in milking frequency may be achieved by altering cell number (Wilde et al, 1987b). Decreasing the level of apoptosis during declining lactation may prolong epithelial cell life and function. Decreased apoptosis during declining lactation may ensure some cells do not die at the end of a lactation cycle and instead these cells may be carried over into a subsequent lactation cycle to increase milk yield in the next lactation.

This thesis has established that many of the extracellular factors necessary for successful lactation are also important regulators of mammary apoptosis. Milk accumulates within alveolar spaces when milk synthesis is greater than demand,and results in a local stimulation of the apoptotic pathway (Chapter 3). Endocrine signals released during suckling maintain lactation (Knight, 1993; Flint et al., 1992) and also act as cell survival factors by suppressing entry of cells into apoptosis (Chapter 4). In addition, in rodents where prolactin is considered the principal galactopoietic hormone,

growth hormone has also been demonstrated to exert an influence on mammary apoptosis (Chapter 4).

The mammosphere culture system has already been used to provide information on the regulation of lactation. The effects of luminal evacuation and filling can be investigated by either EGTA treatment or by microinjection techniques which provide a means of removing and examining mammosphere contents and of adding components to luminal contents and later examining the effect. These techniques will enable a finer tuning of mammary epithelial cells than is possible *in vivo,* and the precise effects of addition of FIL, antibodies to FIL on mammopshere structure and function could be determined. Similarly the mechanism of action of other agents which act locally to induce apoptosis, such as glucocorticoids and progesterone (Feng et al., 1995) can be determined using the EHS culture system. These components could be added alone or in combination and the effects of administration on the amount and composition of milk secretion can be determined by Bradford protein determination and Western blotting for various milk proteins. The effects of these treatments on cell life and death could also be investigated using DNA fragmentation techniques.

Integration between local and endocrine control mechanisms is likely since unilateral teat sealing in mice and unilateral cessation of milking in goats results in increased apoptosis in treated and in untreated tissue (Chapters 3 and 5). Evidence for interaction between local and endocrine control mechanisms has also been obtained from study of the feedback inhibitor of lactation (FIL), since FIL can regulate hormone receptor number in the mammary gland (Bennett, 1993). Comparision of the extent and time course of apoptosis with and without administration of antibody to FIL or antibody to FIL receptors during involution induced by bromocriptine and / or anti

growth hormone treatment may demonstrate the existence of local and endocrine interactions. Alternatively, comparision of the extent and time course of apoptosis and involution with and without administration of prolactin and / or growth hormone during unilateral teat sealing in rodents or during unilateral cesation of milking in ruminants may reveal the extent of local and endocrine interactions. Finally evidence for apoptosis or involution after administration of antibody to FIL or FIL receptors during locally induced apoptosis (teat sealing in rodents mr cessation of milking in ruminants) would enable investigation of the overlap of local and endocrine pathways.

Degeneration of mammary epithelium during gland involution coincided with an increase in both *p53* gene expression and extracellular protease levels (Lefebvre et al., 1992; Strange et al., 1992; Talhouk et al., 1992; Guenette et al., 1994)). Indeed, apoptotic tissue degeneration is frequently associated with elevation of *p53* (Clarke et al., 1993; Lowe et al., 1993) and proteases (Sensibar et al., 1990; Leah et al., 1993), suggesting a common mechanism for apoptosis in many tissues. The timecourse of induction of *p53* and protease activity in mammary tissue depended upon the method of induction of apoptosis, but in most cases both *p53* and protease activity increased simultaneously (Chapter 3). The increase in protease activity suggests mammary apoptosis is dependent on loss of basement membrane integrity. However nick end labelling detected DNA laddering at peak lactation when alveoli were apparently intact (Chapter 3). This suggests that there is not always a link between ECM degradation and apoptosis and that dissociation from the ECM may be sufficient to cause individual cell death. Indeed, *in vitro* lumen formation (Chapter 6) is consistent with a link between ECM contact and cell death, since protease degradation is not imperative to the stimulation of apoptosis and that perhaps loss of contact between intact basement

membrane and the mammary epithelial cells may be sufficient to trigger apoptosis.

To follow up the importance of loss of basement membrane contact and the role of proteases in the mammary gland the effect of a range of proteases and protease inhibitors could be investigated *in vivo* throughout pregnancy, lactation and involution. This may be achieved by either intra-mammary injection of antibodies to proteases and their inhibitors or by intra-mammary implantation of slow release capsules containing proteases and inhibitors. Simliar experiments could be performed with antibodies to or capsules containing basement membrane components to provide comprehensive information on the roles of specific basement membrane components during various phases of the lactation cycle. To date a role for laminin has been determined during ß casein production (Streuli et al 1993), but as yet the precise roles of collagen, vitronectin and fibronectins are undetermined through pregnancy, lactation and involution.

Changes in gene expression have, of course,been catalogued in a wide range of apoptotic situations (Schneider et al., 1988; Strange et al., 1992) and a common pattern of changes in gene expression is emerging (Nunez et al., 1990; Kastan et al.,1991; Yonnish-Rouach et al., 1991; Evan et al., 1992, Fandini et al., 1992; Garcia et al., 1992). As part of my study of mammary apoptosis I have mapped the changes in key genes implicated in programmed cell death and remodelling of the extracellular matrix. Knowledge of the genetic changes will be of central importance in elucidating the apoptotic process in mammary tissue. Therefore in continuation of this project the effect of genes previously shown to be important during apoptosis could be investigated using transgenic studies where a particular gene is selectively over or under expressed in mammary tissue. The effect of transgenes on mammary development, lactation and

involution would determine the gene's role (if any) in mammary apoptosis. Gene interactions could also be identified since omission or enhancement of a gene may alter levels of expression of other genes known to be important during mammary apoptosis. This type of study would provide valuable information on the influence of genetic events during mammary apoptosis.

This transgenic approach to the study of mammary cell turnover is already being exploited in several laboratories. The role of *p53* would be of particular interest during mammary apoptosis since elevation of *p53* is one of the earliest genetic alterations detected during mouse mammary involution (Strange et al., 1992). However mutant *p53* transgenic mice display an impaired ability to lactate and the mice expressing the highest levels of mutant *p53* were unable to nurse their young (Li et al., 1994). In this respect the role of wild type *p53* would be interesting to follow. There is increasing evidence that defective apoptotic cell death may also be an early event in neoplastic development of the mammary gland, and *p53* is reported to be mutated in a high proportion of breast tumours (Levine et al., 1991). The importance of hyperplastic pre-cancerous lesions in human breast cancer has long been recognised (Ewing, 1914). They represent the earliest identifable intermediates in breast tumour development (Page and Dupont, 1990) and their subsequent progression to tumours has been clearly documented (Medina, 1988). If hyperplastic nodules are the product of impaired apoptosis this suggests that the cycle of mammary development and involution during pregnancy, lactation and weaning may confer protection against a malfunction of apoptosis. This may explain why null parity is a positive risk factor for premenopausal mammary cancer. Investigation of this phenomenon warrents further study.

Studies using transgenic animals will also enable the importance of *bcl-2* family genes to be established during mammary apoptosis. This work could ascertain the importance of these genes during mammary development and involution, and establish which members of this gene family are playing a role in programmed cell death not only in the mammary gland but in other tissues, as at present the roles of the different *bcl-2* family genes are unclear (Boise et al., 1993; Gonzalez-Garcia et al., 1994; Oltvai et al., 1993). Animals expressing different *bcl-2* transgenes may show that one family member can substitute for others, or may determine that each member has a clearly defined role in the mammary gland. The precise interactions of the *bcl-2* family with each other (Oltvai et al., 1993; Takayama et al., 1995; Yang et al., 1995) and with other genes which are involved in regulating mammary apoptosis, such as proteases and *p53,* (Wang et al., 1993; Chiou et al., 1994) will increase understanding of the mechanisms underlying the regulation of mammary cell survival and apoptosis.

The information contained in this thesis will, it is hoped, stimulate future studies on intracellular pathways and nuclear events that are important during mammary apoptosis, so that the mechanisms dictating cell survival and apoptosis can be understood. These studies may eventually enable the controlled manipulation of apoptotic pathways in mammary tissue and specific treatment of disease states where apoptotic pathways are malfunctioning.

BIBLIOGRAPHY

Adams, J.C. & Wattt, F.A. (1993). Regulation of development and differentiation by extracellular matrix, *Development,* **117,** 1137-1146.

Afanasev, V.N., Korol, B.A., Mantsygin, Y.A., Nelipovio, P.A., Pechatnikov, V. A. & Urmansky, S.R. (1986). Flow cytometry and biochemical analysis of DNA degradation charcteristic of two types of cell death, *FEBS Lett.*, **194,** 347-348.

Aggeler, J., Ward, J., Blackie, L.M., Barcellos-Hoff, M.H., Streuli, C.H. & Bissell, M.J. (1991). Cytodiferentiation of mouse mammary epithelial cells cultured on a reconstituted basement membrane reveals striking similarities to development in vivo, *J.Cell Biol.*, **99,** 407-417.

Akers, R.M. & Heald, C.W. (1978). Stimulatory effect of prepartum milk removal on secretory cell differentiation in the bovine mammary gland, *J. Ultrastruct. Res.*, **63,** 316-324.

Akers, R.M. (1985). Lactogenic hormones: binding sites, mammary growth, secretory cell differentiation and milk biosynthesis in ruminants, *J. Dairy Sci.*, **68,** 501-516.

Alexander, C.M. & Werb, Z. (1991). Extracellular matrix degradation. In *cell biology of the extracellular matrix,* second edition (ed Hay, E.D.), 255-302, Plenum Press, London.

Allan, D.J., Harmon, B.V., & Kerr, J.F.R. (1987). Cell death in spermatogenesis, In *Perspectives on mammalian cell death,* (ed Potten, C.S.), 229. Oxford University Press, England.

Alnemri, E.S., & Litwack, G. (1990). Activation of internucleosomal DNA cleavage in human CEM lymphocytes by glucocorticoid and Novobiocin, *J. Biol. Chem.*, **265,** 17323-17333.

Anderson, R.R. (1978). Embryonic and fetal development of the mammary apparatus, In *lactation,* (ed Larson, B.L.), Academic Press, New York, **4,** 3-40.

Anderson, R.R., Harness, J.R., Snead, A.F. & Salah, M.S. (1981). Mammary growth pattern in goats during pregnancy and lactation, *J. Dairy Sci.*, **64,** 427-433.

Anderson, R.R., Salah, M.S., Harness, J.R. & Snead, A.F. (1982). Mammary growth patterns in guinea pigs during puberty, pregnancy and lactation, *Biology of reproduction,* **26,** 620-627.

Ansari, B., Cotes, P.J., Greenstein, B.D. & Hall, P.A. (1993). *In situ* end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states,

J. Pathol., **170**, 1-8.

Arends, M.J., Morris, R.G. & Wyllie, A.H.(1990). Apoptosis. The role of the endonuclease, *Am. J. Pathol.*, **136**, 593-608.

Ashkenas, J., Myers, C.A., Schmidhauser, C., Werb, Z. & Bissell, M. (1995). STAT5 activation in mammary epithelial cells cultured with and without ECM, *Mol. Biol. Cell,* **6,** 2228-2234.

Atwood, C.S., Ikeda, M. & Vonderhaar, B.K. (1995). Involution of mouse mammary glands in whole organ culture: a model for studying programmed cell death, *Biochem. and Biophys. Res. Comm.,* **207,** 860-867

Barak, Y. & Oren, M. (1992). Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53 mediated growth arrest, *The EMBO J.*, **11**, 2115-2121.

Barak, Y., Juven, T., Haffner, R. & Oren, M. (1993). mdm-2 expression is induced by wild type p53 activity, *The EMBO J.*, **12,** 461-468.

Barber, M.C., Travers, M T., Finley, E., Flint, D.J & Vernon, R.G. (1992). Growth hormone prolactin interactions in the regulation of mammary adipose tissue acetyl CoA carboxylase activity and gene expression in lactating rats, *Biochem. J.*, **285,** 469-276.

Barcellos-Hoff, M.H., Aggeler, J., Ram, T.G. & Bissell, M.J. (1989). Functional differentiation and alveolar morphogenesis of primary mammary cultures on reeconstituted basement membrane, *Development,* **105**, 223-235.

Barry, M.A. & Eastman, A. (1993). Identification of deoxyribonuclease II as an endonuclease involved in apoptosis, *Arch. Biochem. Biophys.*, **300**, 440-450.

Bauman, D.E., Eppard, P.J., Degeeter, M.J. & Lanza, G.M. (1985). Responses of high producing dairy cows to long term treatment with pituitary somatotrophin in the ruminant, *J. Dairy Sci,* **68,** 1352-1360.

Bchini, O., Andres, A.C., Schubar, B., Mehtali, M., LeMeur, M., Lathe, M. & Gerlinger, P. (1991). Precocious mammary gland development and milk protein synthesis in transgenic mice ubiquitously expressing growth hormone, *Endocrinology,* **128,** 539-546.

Bennett, C.N. (1993). Autocrine mechanisms modulating endocrine regulation of mammary gland function, *PhD. thesis submitted to Glasgow University,* **Chapter six**, 68-74.

Bielke, W., Gou, K., Strange, R. & Friis, R.R. (1995). Apoptosis in mouse mammary gland involution: Isolation and characterisation of apoptosis specific genes In *Intercellular signalling in the mammary gland,* (eds, Wilde, C.J., Peaker, M & Knight, C.H.), Plenum Press, London. 45-56.

Bissell, M.D., Arenson, M.D. & Roll, J.F. (1987). Support of cultured hepatocytes by a laminin rich gel, *J. Clin. Invest.*, **79,** 801-813.

Bissell, M.J. & Barcellos-Hoff, M.H. (1987). The influence of extracellular matrix on gene expression: is structure the message?, *Development,* **105,** 223-235.

Bissonnette, R.P., Echeverri, F., Mahboubi, A. & Green, D.R. (1992). Apoptotic cell death induced by c myc is inhibited by bcl-2, *Nature*, **359**, 552-554.

Blatchford, D.R. & Peaker, M. (1982). Effects of frequent milking on milk secretion during lactation in the goat: Relation of factors which limit the rate of secretion, *Q. J. Exp. Physiol.*, **67**, 303-310.

Blatchford, D.R., Hendrey, K.A.K., Turner, M.D., Burgoyne, R.D. & Wilde, C.J. (1995). Vectorial secretion by constitutive and regulated secretory pathways in mammmary epithelial cells, *Epith.Cell Biol.*, **4,** 8-16.

Boise, L.H., Gonzalez Garcia, M., Postema, C.E., Ping, L., Lindsten, T., Turka, L.A., Mao, Y., Nunez, G. & Thompson, C.B. (1993). Bcl-x, a bcl-2 related gene that functions as a dominant regulator of apoptotic cell death, *Cell*, **74**, 597-608.

Boudreau, N., Sympson, C.J., Werb, Z. & Bissell, M.J. (1995). Supression of ICE and apoptosis in mammary epithelial cells by extracellular matrix, *Science,* **267,** 891-893.

Brandes, D., Anton, E. & Barnard, S. (1969). Lysosomes and cellular regressive changes in rat mammary gland involution, *Lab. Clinical Invest.*, **20,** 465-471.

Bresciani, F. (1971). Ovarian steriod content of cell proliferation in the mammary gland and cancer, In *basic actions of sex steriods on target organs* (ed Hubinont, P.O., Leroy, F. & Galand, P.) p130-159.

Brown, D.G., Sun, X.M. & Cohen, G.M. (1993). Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to intranucleosomal fragmentation, *J. Biol. Chem.*, **268,** 3037-3039.

Buttle, D.J., Handley, C.J., Ibic, M.Z., Saklatuala, J., Murata, M. & Barrat, A.J. (1993). Inhibition of cartilage proteoglycan release by a specific inactivator of cathepsin ß and an inhibitor of matrix metalloproteinases - evidence for two converging pathways of chonndrocyte - mediated proteoglycan degradation, *Arthritis and Rheumatism,* **36,** 1709-1717.

Carlsson, E.L., Karlsson, B.W. & Waldemarson, K.H.C. (1973). Dehydrogenases and nucleic acids in rat mammary gland during involution initiated at various stages of lactation, *Comp, Biochem, Physiol,* **44,** 93-108.

Chang, A.C., Solomon, D.R., Wadsworth, S., Hong, M.P., Mojcik, C.F.,Otto, S.,

Sheach., E.M. & Coligan, J.E. (1995). α3ß1 and α6ß1 integrins mediate laminin / merosin binding and function as costimulatory molecules for human thymocyte proliferation, *The Journal of Immunology,* **154,** 500-510.

Chen, L.H. & Bissell, M.J. (1989). A novel regulatory mechanism for the whey acidic protein gene expression, *Cell Regulation,* **1,** 45-54.

Chen, C., Oliner, J.D., Zhan, Q., Fornace, A.J., Vogelstein, B. & Kastan, M.B. (1994). Interactions between p53 and mdm-2 in a mammalian cell cycle checkpoint pathway, *Proc. Natl. Acad. Sci.*, **91**, 2684-2688.

Chiou, S., Rao, L. & White, E. (1994). Bcl-2 blocks p53 dependent apoptosis, *Mol. Cell. Biol.*, **14**, 2556-2563.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, *Biochemistry,* **18,** 5294-5299.

Clarke, A.R., Purdie, C.A., Harrison D.J., Morris, R.G., Bird, C.C., Hooper, M.L. & Wyllie, A.H., (1993). Thymocyte apoptosis induced by *p53*-dependent and independent pathways, *Nature,* **362,** 849-852.

Cohen, J.J. & Duke, R.C. (1984). Glucocorticiod activation of a calcium dependent endonuclease in thymocyte nuclei leads to cell death, *J. Immunol.,* **132,** 38-42.

Collier, R.J., McGrath, M.F., Byatt, J.C. & Zurfluh, L.L. (1993). Regulation of mammary growth by peptide hormones: involvement of receptors, growth factors and bindinng proteins, *Livestock Production Science,* **34,**21-28.

Collier, R.J., Byatt, J.C., McGrath, M.F. & Eppard, P.J. (1995). Role of bovine placental lactogen in intracelluar signalling during mammary growth and lactation, In *Intracellular signaling in the mammary gland,* (eds Wilde, C.J., Peaker, M. & Knight, C.H.), 13-24, Plenum Press, London.

Collins, R.J., Harmon, B.V., Gobe, G.C. & Kerr, J.F.R. (1992). Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis, *Int. J. Radiat. Biol.*, **61**, 451-453.

Colombel, M., Olsson, C.A., Ng, P. & Buttyan, R. (1992). Hormone regulated apoptosis results from reentry of differentiated prostate cells onto a defective cell cycle, *Cancer Research*, **52**, 4313-4319.

Compton, M.M., Haskill, J.S. & Cidlowski, J.A. (1988). Analysis of glucocorticoid actions on rat thymocyte deoxyribonucleic acid by fluorescence-activated flow cytometry, *Endocrinology*, **122**, 2158-2164.

Cowie, A.T. (1949). The relative growth of the mammary gland in normal,

gonadectomized and adrenalectomized rats, *J. Endocrinol.*, **6,** 146-157.

Cowie, A.T., Knaggs, G.S. & Tindall, J.S., (1964). Complete restoration of lactation in the goat after hypophysectomy, *J.Endocrinol,* **28,** 267-274.

Cowie, A.T., Forsyth, I.A. & Hart, I.C. (1980). In *Hormonal Control of Lactation*, Spriner-Verlag, Berlin.

Deeks, S., Richards, J. & Nandi, S. (1988). Maintenence of normal rat epithelial cells by insulin and insulin like growth factor 1, *Experimental Cell Res.*, **174,** 448-460.

Del Sal, G., Ruaro, M.E., Philipson, L. & Schneider, C. (1992). The growtyh arrest specific gene gas-1 is involved in growth suppression, *Cell,* **70,** 595-607.

Diller, L., Kassel, J., Nelson, C.E., Gryka, M.A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S.J., Vogelstein, B. & Friend, S.H. (1990). p53 functions as a cell cycle control protein in osteosarcomas, *Mol. Cell. Biol.*, **10**, 5772-5781.

Docherty, A.J.P., Lyons, A., Smith, B.J., Wright, E.M. Stephens, P.E., Harris, T. J.R., Murphy, G. & Reynolds, J.J. (1985). Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity, *Nature,* **318,** 68-69.

Drago, J., Murphy, M., Carroll, S.M., Harvey, R.P. & Bartlett, P.F. (1991). Fibroblast growth factor mediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor 1, *Proc, Natl. Acad. Sci.*, **88,** 2199-2203.

Duke R.C., Cohen, J.J. & Chervenak, R. (1986). Differences in target cell DNA fragmentation induced by mouse cytotoxic T lymphocytes and natural killer cells, *J. Immunol.*, **137,** 1442-1447.

Duvall, E. & Wyllie, A H. (1986). Death and the cell, *Immunol. Today,* **7,** 115-121.

Eastman, A. (1990). Activation of programmed cell death by anticancer agents: cisplatin as a model system, *Cancer Cells*, **2**, 275-280.

Edwards, G. & Streuli, C. (1995). Signalling in extracellular-matrix mediated control of epithelial cell phenotype, *Biochem. Soc. Trans.*, **23,** 464-468.

Eleftheriou, E.P. (1986). Ultrastructural studies on protophloem sieve elements in *Triticum aestivuro L.* Normal degradation, *J. Ultrastruct. Mol. Struct. Res.*, **95**, 47.

Ellis, R., Yuan, J. & Horvitz, H.R. (1991). Mechanisms and functions of cell death, *Ann. Rev. Cell. Biol.* **7**, 663-698.

Evan, G.I, Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Broks, M., Waters, C.M., Penn, L.Z & Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein, *Cell,* **69,** 119-129.

Ewing, J. (1914). *Medical Records,* **86,** 951.

Fanidi, A., Harrington, E.A. & Evan G.I. (1992). Cooperative interaction between c myc and bcl-2 proto oncogenes, *Nature*, **359**, 554-556.

Feinberg, A.P. & Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, *Analytical Biochem.*,**132,** 6-13.

Feng, Z., Marti, A., Jehn, B., Altermatt, H.J., Chicaiza, G. & Jaggi, R. (1995). Glucocorticioid and progesterone inhibit involution and programmed cell death in the mouse mammary gland, *J. Cell Biol.*, **131,** 1095-1130.

Ferguson, D.J.P. & Anderson. T.J. (1981). Morphological evaluation of cell turnover in relation to the menstrual cycle in the "resting" human breast, *Br. J. Cancer*, **44**, 177-181.

Fidzianska, A., Goebel, H.H. & Warlo, I. (1990). Acute infantile spinal muscular atrophy: Muscular apoptosis as a proposed pathogenic mechanism, *Brain*, **113**, 433-445.

Fleet, I.R., Goode, J.A., Hamon, M.H., Lawrie, M.S., Linzell, J.L. & Peaker, M. (1975). Secretory activity of goat mammary glands during pregnancy and the onset of lactation, *J. Physiol.*, **251,** 763-773.

Flint, D.J., Tonner, E., Beattie, J. & Panton, D. (1992). Investigation of the mechanism of action of growth hormone in stimulating lactation in the rat, *J. Endocrinol.*, **134,** 337-383.

Flint, D.J. & Gardiner, M. (1994) Evidence that growth hormone stimulates milk synthesis by direct action on the mammary gland aand that prolactin exerts effects on milk secretion by maintenence of mammary deoxyribonucleic acid content and tight junction status. *Endocrinology,* **135,** 1119-1124.

Flint, D.J., Tonner, E., Beattie, J., & Panton, D. (1994). Several insulin like growth factor-1 analogues and complexes of insulin like growth factors 1 and 2 with insulin like growth factor binding protein 3 fail to mimic the effect of growth hormone upon lactation in the rat, *J. Endocrinol.*, **140,** 211-219.

Flint, D.J. (1995). Regulation of milk secretion and composition by growth hormone and prolactin, In *Intercellular signaling in the mammary gland,* (eds Wilde, C.J., Peaker, M. & Knight, C.H.), 131-141, Plenum Press, London.

Fornace, A.J., Nerbert, D.W., Hollander, M.C., Luethy, J.D., Papathanasiou, M.,

Fargnoli, J. & Holbrook, N.J. (1989). Mammalian genes coordinately regulated by growth arrest signals and DNA damaging agents, *Mol. Cell. Biol.*, **9**, 4196-4203.

Forsyth, I.A. & Jones, E.A. (1976). Organ culture of mammary gland and placenta in the study of hormone action and placental lactogen secretion. In *organ culture in biomedical research,* (eds Balls, M. & Monnickendam, M.A.), 201-221. Cambridge University Press.

Forsyth, I.A. (1986). Variation amongst species in the endocrine control of mammary growth and function: the roles of prolactin, growth hormone, and placental lactogen, *J. Dairy Sci.*, **69,** 886-893.

Foster, R.C. (1977). Changes in mouse mammary epithelial cell size during mammary gland development, *Cell Diff.*, **6,** 1-8.

Franks, S., Kiwi, R. & Nabarro, J.D.N. (1977). Pregnancy and lactation after pituitary surgery, *Br. Med. J.*, **1,** 882.

Garcia, I., Marinou, I., Tsujimoto, Y. & Martinou, J,C. (1992). Prevention of programmed cell death of sympathetic neurones by the bcl-2 proto-oncogene, *Science*, **258**, 302-304.

Gavrieli, Y., Sherman, Y. & Ben Sasson, S.A. (1992). Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation, *J. Cell. Biol.*, **119**, 493-501.

Gaido, M.L. & Cidlowski, J.A. (1991). Identification, purification and characterisation of a calcium dependent endonuclease (NUC18) from apoptotic rat thymocytes: NUC18 is not histone H^2B, *J. Biol. Chem.*, **266**, 18580-18585.

Gewert, D.R., Coulombe, B., Castelino, M., Skup, D & Williams, B.R.G. (1987). Characterisation and expression of a murine gene homologous to human EPA/TIMP: a virus-induced gene in the mouse, *EMBO J.*, **6,** 651-657.

Ginn, F.L., Hochstein, P. & Trump, B.F. (1969). Membrane alterations in heamolysis: Internalization of plasmalemma induced by primaquine, *Science,* **164,** 843-845.

Glucksmann, A. (1950). Cell death in normal vertibrate ontogeny, *Biol. Rev. Cambridge Philos. Soc.*, **26**, 59-86.

Goodman, R.E. & Schanbacher, F.L. (1991). Bovine lactoferrin mRNA: sequence, analysis and expression in the mammary gland, *Biochem. Biophys. Res. Comm.*, **180,** 175-179.

Gonzalez-Garcia, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L. H., Thompson, C.B. & Nunez, G. (1994). bcl-xl is the major bcl-x mRNA form expressed during murine development and its product localises to mitochondria, *Development,*

120, 3033-3042.

Graham, R.E. & Bertalanffy, F.D. (1972). Cell division in normal and neoplastic mammary gland tissue in the rat, *Anat. Rec.*, **174,** 1-7.

Groner, B. and Gouilleux, F. (1995). Prolactin-mediated gene activation in mammary epithelial cells, *Current Opinion in Genetics and Development, 5,* 587-594.

Griffith, D.R. & Turner, C.W. (1961). Normal growth of rat mammary glands during pregnancy and early lactation, *Proc. Soc. exp. Biol. Med,* **106,** 448-450.

Griffith, D.R. & Turner, C.W. (1962). Hormonal control of mammary gland involution in the rat, *Proc. Soc. Exp. Biol. Med.*, **110,** 485-487.

Guenette, R.S., Corbeil, H.B., Leger, J., Wong, K., Mezl, V., Mooibroek, M. & Tenniswood, M. (1994). Induction of gene expression during involution of the lactating mammary gland of the rat, *J. Mol. Endo.*, **12**, 47-60

Guenette, R.S. & Tenniswood, M. (1995). The role of insulin like growth factor binding proteins (IGFBP's) in regulating active cell death in regressing rat prostate and mammary gland, *J. Cel Biochem. Supp.*, **19B,** 280.

Guyette, W.A., Matusik, R.J. & Rosen J.M. (1979). Prolactin mediated transcriptional and post transcriptional control of casein gene expression, *Cell,* **17,** 1013-1019.

Hall, D.E., Richardt, L.F., Crowley, E., Hooley, B., Moezzi, H., Sonneenberg, A. & Damsky, C.H. (1990). The alpha 1/beta 6 and alpha 6/beta 1 integrin heterodimers mediate cell attatchment to distinct sites on laminin, *J. Cell Biol.*, **110,** 2175-2184.

Hart, I.C. & Flux, D.S. (1973). The release of growth hormone in response to milking in the goat during early and late lactation. *J. Endocrinol.*, **57**, 177-178

Hauser, S.D., McGrath, M.F., Collier, R.J. & Krivi,G.G. (1990). Cloning and in vivo expression of bovine growth hormone receptor mRNA, *Mol. Cell Endocrinol, 72,* 187-193.

Hawkins, H.K., Ericsson, J.L., Binerfeld, P. & Trump, B.F. (1972). Lysosome and phagosome stability in lethal cell injury. Morphologic tracer studies in cell injury due to inhibition of energy metabolism, immune cytolysis and photosensitisation, *Am. J. Pathol.*, **68,** 255-258.

Hayden, T.J., Thomas, C.R. & Forsyth, I.A. (1979). Effect and number of young born (litter size) on milk yield of goats: a role for placental lactogen, *J. Dairy Sci,* **62,** 53-63.

Hekman, C.M. & Loskutoff, D.J. (1985). Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants, *J. Biol. Chem.*, **260**,

11581-11587.

Helminen, H.J. & Ericsson, J.L.E. (1968). Studies on mammary gland involution, *J. Ultrastruct. Res.*, **25,** 193-239.

Helminen, H.J., Ericsson, J.L.E. & Orrenius, S. (1968). Histochemical and biochemincal observations on alterations in lysosomes or lysosymal enzymes, *J. Ultrastruct, Res., 25,* 240-252.

Henderson, A.J., Blatchford, D.R. & Peaker, M. (1983). The effects of milking thrice instead of twice daily on milk secretion in the goat, *Q. J. Exp. Physiol.*, **68**, 645-652.

Henderson, A.J. & Peaker, M. (1984). Feedback control of milk secretion in the goat by a chemical present in milk, *J. Physiol.*, **351**, 39.

Henderson, A.J., Blatchford, D.R. & Peaker, M. (1985). The effects of long term thrice daily milking on milk secretion in the goat: evidence for mammary growth, *Q. J. Exp. Physiol.*, **70**, 557.

Henson, D.E. & Tarone, R.E. (1994) Involution and the etiology of breast cancer, *Cancer,* **74,** 424-429.

Hockenbery, D.M., Zutter, M., Hickey, W., Nahm, M., & Korsmeyer, S.J. (1991). Bcl-2 protein is topographically restricted in tissues characterised by apoptotic cell death, *Proc. Natl. Acad. Sci. USA*, **88**, 6961-6965.

Howlett, A.R. & Bissell, M.J. (1993). The influence of tissue microenvironment (Stroma and extracellular matrix) on the development and function of mammary epithelium, *Epithel. Cell Biol., 2,* 79-89.

Hurley, W.L.(1989). Mammary gland function during involution, *J. Dairy Sci.*, **72,** 1637-1646.

Ichinose, R.R. & Nandi, S. (1964). Lobuloalveolar differentiation in mouse mammary tissue *in vitro, Science,* **145,** 496-497

Ichinose, R.R. & Nandi, S. (1966). Influence of hormones on lobuloalveolar differentiation of mouse mammary glands *in vitro, J. Endocrinol,* **35,** 331-340.

Iseki, R., Mukai, M. & Iwata, M. (1991). Regulation of T lymphocyte apoptosis. Signals for the antagoism between activation- and glucocorticiod- induced death, *J. Immunol.*, **147**, 4286-4292.

Ip, M.M. & Darcy, K.M. (1996). Three dimensional mammary primary culture model systems, *J. Mammary Gland Biol. and Neoplasia,* **1,** 91-110.

Jacks, T., Remington, L., Williams, B., Schmitt, E., Halachini, S., Bronson, R. &

Ilkbahar, Y.N., Wu, K., Thordarson, G. & Talamantes, F. (1995). Expression and distribution of mRNA's for growth hormone receptor and growth hormone binding protein in mice during pregnancy, *Endocrinology,* **136,** 386-392.

Jammes, H., Gaye, P., Belair, L. & Djiane, J. (1991). Identification and characterisation of growth hormone receptor mRNA in the mammary gland, *Mol. and Cell. Endocrinol.*, **75**, 27-35.

Weinberg, R. (1994). Tumor spectrum analysis in p53 mutant mice, *Curr. Biol.*, **4**, 1-7.

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C. & Raff, M.C. (1993). Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA, *Nature*, **361**, 365-369.

Jennings, R.B. & Reimer, K.A. (1981). Lethal myocardial ischemic injury, *Am. J. Pathol.*, **102**, 241-255.

Jerry, J.D., Ozbun, M.A., Kittrell, F.S., Lane, D.P., Medina, D. & Butel, J.S. (1993). Mutations in p53 are frequent in the preneoplastic stage of mouse mammary tumour development, *Cancer Res.*, **53**, 3374-3381.

Jones, E.A. (1967) Changes in the enzyme pattern of the mammary gland of the lactating rat after hyperphysectomy and weaning, *Biochem. J.*, **103**, 420-427.

Karg, H., Schams, D. & Reinhardt, U. (1972). Effects of 2-Br-α-ergocryptone on plasma prolactin level and milk yield in cows, *Experimentia*, **28**, 574-576.

Kastan, M.B., Radin, A.I., Kuerbitz, S.J., Onyekwere, O., Wolkow, C.A., Civin, C.T., Stone, K.D., Woo,T., Ravindranath, Y. & Craig, R.W. (1991). Levels of p53 protein increase with maturation in human hematopoietic cells, *Cancer Res.*, **51**, 4279-4286.

Kastan, M.B., Zhan, Q.M., Eldeiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., Fornace, A.T. (1992). A mammalian cell cycle checkpoint pathway utilising p53 and GADD45 is defective in ataxia-telangectasia, *Cell*, **71**, 587-597.

Kazansky, A.V., Raught, B., Lindsey, S.M., Wang, Y.F. & Rosen, J.M. (1995). Prolactin and interleukin receptors in T lymphocytes signal through development, *Mol. Endocrinol.*, **9**, 1598-1609.

Kelly, P.A., Tsushima, T.M., Shui, R.P.C. & Friesen, H.G. (1976). Lactogenic and growth hormone like activities in pregnancy determined by radioreceptor assays, *Endocrinology*, **99**, 765-774.

Kern, S.E., Kinzler, K., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., Vogelstein, B. (1991). Identification of p53 as a sequence specific DNA binding protein, *Science*, **252**, 1708-1711.

Kerr, J.F.R. (1965). A histochemical study of hypertrophy and ischaemic injury of rat liver with special reference to changes in lysosomes, *J. Pathol. Bacteriol.*, **90**, 419.

Kerr, J.F.R. (1971). Shrinkage necrosis. A distinct mode of cellular death, *J. Pathol.*, **105**, 13-23.

Kerr, J.F.R., Wyllie, A.H. & Currie, A.R. (1972). Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics, *Br. J. Cancer,* **26,** 239-246.

Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W. & Martin, G.R. (1986). Basement membrane complexes with biological activity, *Biochemistry,* **25,** 312-318.

Klonner, R.A., Ganote, C.E., Whalen, D.A. & Jennings, R.B. (1974). Effect of a transient period of ischemia on myocardial cells: Fine structure during the first few minutes of reflow, *Am. J. Pathol, 74,* 399-422.

Knight, C.H. & Peaker, M.(1982a). Development of the mammary gland, *J. Reproduction and Fertility,* **65,** 521-536.

Knight, C.H. & Peaker, M. (1982b). Mammary cell proliferation in mice during late pregnancy and lactation in relation to milk yield, *Q. J. Physiol.*, **67,** 165-177.

Knight, C.H., Maltz, E. & Peaker, M. (1983). A simple technique for estimating milk yield in rats and mice, *J. Physiol.*, **336,** 73-74.

Knight, C.H. & Peaker, M. (1984). Mammary development and regression during lactation in goats in relation to milk secretion, *Q. J. Exp. Physiol.*, **69**, 331-338.

Knight, C.H., Docherty, A.H. & Peaker, M. (1984). Milk yield in rats in relation to activity and size of the mammary secretory cell population, *J.Dairy Res.*, **51,** 29-35.

Knight, C.H., Maltz, E. & Docherty, A.H., (1986). Milk yield and composition in mice: effects of litter size and lactation number, *Comp. Biochem. Physiol.*, **84A,** 127-133.

Knight, C.J. & Wilde, C.J. (1987). Mammary growth during lactation: Implications for increasing milk yield, *J. Dairy Sci.*, **70**, 1987-1991.

Knight, C. H. & Wilde, C.J. (1988). Milk production in concurrently pregnant and lactating goats mated out of season, *J.Dairy Res.*, **55,** 487-493.

Knight, C.H., Fowler, P.A. & Wilde, C.J. (1990). Galactopoietic and mammogenic effects of long term treatment with bovine growth hormone and thrice daily milking in goats, *J.Endocrinol., 127,* 129-141.

Knight, C.H. (1993). Prolactin revisited. *Hannah Research Institute Yearbook 1993*, (ed, Taylor, E.), Thompson, Glasgow, 72-80.

Krajewski, S., Krajewska, M., Shabaik, A., Wang, H.G., Irie, S., Fong, L. & Reed, J.C.(1994). Immunohistochemical analysis of *in vivo* patterns of bcl-x expression. *Cancer Res.*, **54,** 5501-5507.

Kubler, D.M., Jordan, P.W., O'Neill, C.H. & Watt, F.M. (1991). Changes in the adundance and distribution of actin and associated proteins during terminal diferentiation of human keratinocytes, *J. Cell Sci,* **100,** 153-162.

Labarca, C. & Paigen, K., (1980). A simple and sensitive DNA assay procedure, *Anal. Biochem.*, **102,** 344-352.

Laiho, K.V., Berezesky, I.K. & Trump, B.F. (1983). Studies on the modification of the cellular respons to injury - following injury by anoxia or para chloromercuribenzenesulfonic acid (PCMBS), *Experimental and Molecular Pathology,* **1,** 110-128.

Leah, M., Richardson, K.K., Horn, D.M., Wightman, K.M., Hall, R.L., Smith, W.C., Engelhardt, J.A. & Richardson, F.C. (1993). Expression of TRPM-2 during involution and regeneration of the rat liver, *Cancer Letters,* **71,** 133-142.

Ledda Columbano, G.M., Coni, P., Faa, G., Manenti G. & Columbano, A. (1992). Rapid induction of apoptosis in rat liver by cycloheximide, *Am. J. Pathol,* **140,** 545-549.

Lefebvre, O., Wolf, C., Limarcher, J., Hutim, P., Wendling, C., LeMeur, M., Baset, P. & Rio, M. (1992). The breast cancer associated stromelysin 3 gene is expressed during mouse mammary gland apoptosis, *J. Cell Biol.*,**119,** 997-1002.

Letterio, J.J., Geiser, A.G., Kulkarni, A.B., Roche, N.S., Sporn, M.B. & Roberts, A.B. (1994). Maternal rescue of transforming growth factor-ß1 null mice, *Science,* **264,** 1936-1938.

Levine, A.J., Momand, J. & Finlay, C.A. (1991). The p53 tumour supressor gene, *Nature,* **351,** 453-456.

Levine, B., Huang, Q., Isaccs, J.T., Reed, J.C., Griffin D.E. & Hardwick, J.M., (1993). Conversion of lytic to persistant alphavirus infection by the *bcl-2* oncogene, *Nature,* **361,**739-742.

Li, M.L., Aggeler, J., Farson, D.A., Hatier, C., Hassell, J & Bissell, M.J. (1987). Influence of reconstituted basement membrane and its constituents on casein gene expression and secretion in mouse mammary epithelial cells, *Proc. Natl. Acad. Sci. USA,* **84,** 136-145.

Li, B., Greenberg, N., Stephens, C., Meyn, R., Medina, D. & Rosen, J.M. (1994). preferential over expression of 172 Arg - Leu mutant *p53* in the mammary gland of transgenic mice results in altered lobuolalveolar development, *Cell Growth and Differentiation,* **5,** 711-721.

Lin, C.Q., Dempsey, P.J., Coffey, R.J. & Bissell, M.J. (1995). Extracellular matrix regulates whey acidic protein gene expression by suppression of TGF α in mouse

Lockshin, R.A., Alles, A., Kodamen, N., Zakeri, Z.F. (1991). Programmed cell death and apoptosis; early DNA degradation does not appear to be prominent in either embryomic cell death or metamorphosis of insects, *FASEB,* **5,** A518.

mammary epithelial cells: Studies in culture and transgenic mice, *J. Cell Biol.*, **129,** 1115-1126.

Liu, J.K., Deperiso, M.C. & Zarret, K.S. (1991). Extracellular signals that regulate liver transcription factors during hepatic differentiation in vitro, *Mol. Cell Biol.*, **11,** 773-783.

Lui, X.W., Robinson, G.W., Gouillen, F., Groner, B. & Hennighausen, L. (1995). Cloning and expression of STAT5 and an additional homolog (STAT5B) involved in prolactin signal transduction in mouse mammary tissue, *Proc. Natl. Acad. Sci. USA*,**92,** 8831-8835.

Lowe, S.W., Ruley,H.E., Jacks, T. & Housman, D.E. (1993). *P53* dependent apoptosis modulates the cytotoxicity of anticancer agents, *Cell,* **74,** 957-967.

Lucas, M., Solano, F. & Sanz, A. (1991). Induction of programmed cell death (apoptosis) in mature lymphocytes, *FEBS Lett.*, **279**, 19-20.

Lyons, W.R. (1958). Hormonal synergism in mammary growth, *Proc. R. Soc.*, **149,** 303-325.

M^{c}Call, C.A. & Cohen, J.J. (1991). Programmed cell death in terminally differentiating keratinocytes: Role of endogenous nuclease, *J. Invest. Dermatol.*, **97**, 111-114.

M^{c}Grath, M.F., Collier, R.J., Clemmons, D.R., Busby, W.H., Sweeny, C.A. & Krivi, G.G. (1991). The direct *in vitro* effect of insulin-like growth factors (IGFs) on normal bovine mammary cell proliferation and production of IGF binding proteins, *Endocrinology,* **129,** 671-678.

Madon, R.J., Ensor, D.M., Knight, C.H. & Flint, D.J. (1986). Effects of an antiserum to rat growth hormone on the lactating rat, *J. Endocrinol,* **111,** 117-126.

Mao, F.C. & Bremel, R.D. (1990). Development of sensitive α lactalbumin, ß lactalbumin, and αs1 casein ELISA's for mammary explant culture study, *J. Dairy Sci.*, **73,** 241.

Mao, F.C., Bremel., R.D. & Dentine, M.R. (1991). Serum concentrations of the milk proteins α lactalbumin and ß lactalbumin in pregnancy and lactation : correlations with milk and fat yields in dairy cattle, *J. Dairy Sci.*, **74,** 2952-2964.

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

Martin, S.J. (1993). Protein or RNA synthesis inhibition induces apoptosis of mature human CD4+ T cell blasts, *Immulology letters,* **35,** 125-134.

Martinez-Hernandez, A., Fink, L.M. & Pierce, G.B. (1976). Removal of basement membrane in the involuting breast, *Lab. Invest.*, **34**, 455-462.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M.C. & Breathnach, R. (1985). Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts, *EMBO J.*, **4**, 1435-1440.

Matthews, L.H. (1952). In British Mammals, (eds Matthews, L.H. & Harrison, L.), 190, Collins, London.

Medina, D. (1988). The preneoplastic state in mouse mammary tumourigenesis, *Carcinogenesis*, **9**, 1113-1119.

Meites, J., Lu, R.H., Wuttke, W., Welsch, C.W., Nagasawa, H. & Quadri, S.K. (1972). Recent studies on functions and control of prolactin secretion in rats. *Recent Prog. Horm. Res.*, **28**, 471-525.

Michalovitz, D., Halevy, D. & Oren, M. (1990). Conditional inhibition of transformation and of cell proliferation by a temperature sensitive mutant of p53, *Cell*, **62**, 671-680.

Miyashita, T. & Reed, J.C. (1993). Bcl-2 oncogene blocks chemotherapy induced apoptosis in a human leukemia cell line, *Blood*, **81**, 151-157.

Miyashita, T., Krajewski, S., Krajewski, M.,Wang, H.G., Lin, H.K., Leibermann, D.A., Hoffman, B. & Reed, J.C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro and in vivo, Oncogene*,**9**, 1799-1805.

Miyashita, T. & Reed J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, *Cell*, **80**, 293-299.

Monaghan, P., Robertson, D., Amos, A.S., Dyer, M.J.S., Mason, D.Y. & Greaves, M.F. (1992). *J. Histochem. Cytochem.*, **40**, 1819-1825.

Munford, R.E.(1963). Changes in the mammary glands of rats and mice during pregnancy, lactation and involution (ii) levels of deoxyribonucleic acid, and alkaline and acid phosphatases, *J. Endocrinol.*, **28**, 17-34.

Neilson, P.V., Peterson, H. & Kampmann, E.M. (1979). Absence of placental lactogen in an otherwise uneventful pregnancy, *Am. J. Obstet. Gynecol.*, **135**, 322-330.

Nunez, G., Hockenberry, D., McDonnell, T.J., Sorensen, C.M. & Korsmeyer, S.J. (1990). Bcl-2 maintains B cell memory, *Nature*, **353**, 71-73.

Oberhammer, F., Parzefall, W., Breit, P., Erber, E.E., Stadler, M. & Schuite-Herman, R. (1991). Effect of transforming growth factor ß on cell death of cultured rat hepatocytes, *Cancer Res.*, **52**, 2478-2485.

Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R. & Sikroska, M. (1993). Apoptotic death in epithelial cells: cleavage of DNA to 300 and / or 50kb fragments prior to or in the absence of internucleosomal fragmentation, *EMBO J.*, **12,** 3679-3684.

Oltvai, Z.N., Milliman, C.L. & Korsmeyer, S.L. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, bax, that accelerates programmed cell death, *Cell*, **74**, 609-619.

O' Shea, J.D., Hay, M.F. & Cran, D.G. (1978). Ultrastructural changes in the theca interna during follicular atresia in sheep, *J. Reprod. Fert.*, **54**, 183.

Ota, K., Yokoyama. Y. & Schinde, Y. (1962). Effects of administration of oxytocin and prolactin on nucleic acids and phosphoprotein contents of mammary glands in lactating rats, *Nature,* **195,** 77-78.

Ota, K. (1964). Mammary involution and engorgement after arrest of suckling in lactating rats induced by the contents of nucleic acids and milk protein in the gland, *Endocrinol.*, **11,** 146-152.

Paape, M.J. & Sinha, Y.N. (1971). Nucleic acid and collagen content of mammary glands between 30 and 80 days of age in normal and ovarectomized fats and during pregnancy, *J. Dairy Sci,* **54,** 1068-1074.

Page, D.L. & Dupont, W.P. (1990). Anatomic markers of human premalignancy and risk of breast cancer, *Cancer,* **66,** 1326-1335.

Peaker, M. (1989). Evolutionary stratagies in lactation: nutritional implications, *Proc. Nutr. Soc.*, **48,** 53-57.

Peitsh, M.C., Hesterkamp, T., Polzar, B., Mannhez, H.G. & Tschopp, J. (1992). Functional characterisation of serum DNase I in MRL-(lpr) / lpr mice, *Biochem. Biophys. Res. Commun.*, **186**, 739-745.

Pittius, C.W., Hennighausen, L., Lee, E., Westphal, H., Nicols, E., Vitale, J. & Gordon, K. (1988). A milk gene promoter directs the expresion of human - tissue plasminogen activator cDNA to the mammary gland in transgenic mice, *Proc. Natl. Acad. Sci. USA,* **85,** 5874-5878.

Pittius, C.W., Sankaran, L., Topper, Y.J. & Hennighausen, L. (1988). Comparision of the regulation of the whey acidic protein gene with that of a hybrid gene containing the whey acidic protein gene promoter in transgenic mice, *Molecular Endocrinology,* **2,** 1027-1032.

Politis, I., Zavizion, B., White, J.H., Goldberg, J.J., Baldi, A. & Akers, M. (1995). Hormonal and extracellular matrix regulation of plasminogen activator in a bovine epithelial cell line, *Endocrine,* **3,** 345-350.

Quarrie, L. H., Addey, C.V.P. & Wilde, C.J. (1995). Apoptosis in lactating and involuting mouse mammary tissue demonstrated by nick end labelling, *Cell Tissue Res.*, **281,** 413-419.

Potten, C.S. (1977). Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation, *Nature*, **269**, 518.

Prichard, J.A., MacDonald, P.C. & Gant, N.F. (1985). In *Williams Obstetrics*- 17th edition, Appleton Century Crofts, Norwalk.

Radnor, C.J. (1972). Myoepithelial cell differentiation in rat mammary glands, myoepithelium in involuting mammary glands of the rat, myoepithelium in the prelactactating and lactating mammary glands of the rat, *J. Anat.*, **112,** 337-389.

Richards, R.C. & Benson, G.K. (1971). Ultractructural changes accompanying involution of the mammary gland in the albino rat, *J. Endocrinol.*, **51,** 127-135.

Rattray, P.V., GArrett, W.N., East, N.E. & Hinman, N., (1974). Growth, development and composition of the ovine conceptus and mammary gland during pregnancy, *J. Anim. Sci.*, **38,** 613-626.

Robinson, S.D., Silberstein, G.B., Roberts, A.B., Flanders, K.C. & Daniel, C.W. (1991). Regulated expression and growth-inhibitory effects of transforming factor, *Development,* **113,** 867-878.

Rodriguez-Tarduchy G, Collins, M.K.L., Garcia, I. & Lopez-Rivas, A. (1992) Insulin-like growth factor-1 inhibits apoptosis in IL-3 dependent hemopoietic cells, *J. Immunol.*, **149,** 535-540.

Rosen, J.M. & Barker, S.W. (1976). Quantitation of casein messenger ribonucleic acid sequences using a specific complementary DNA hybridisation probe, *Biochemistry,* **15,** 5272-5234.

Rosen, J.M., Jones, W.K., Rodgers, J.R., Compton, J.R. & Brisbee, C.A. (1986). Regulatory sequences involved in the hormonal control of casein gene expression, *Ann. NY Acad. Sci.*, **464,** 87-99.

Ruan, W., Newman, C.B. & Kleinberg D.L. (1992). Intact and amino termainally shortened forms of insulin-like growth factor-1 induce mammary gland diferentiation and development, *Proc. Natl. Acad. Sci. USA.* **89,** 10872-10876.

Sabourin, J.C., Martin, A., Baruch, J., Truc, J.B., Gompel, A. & Poitout, P. (1994). Bcl-2 expression in normal breast tissue during the menstrual cycle, *Int. J. Cancer*, **59**, 1-6.

Sampson, D.A. & Jansen, G.R. (1984). Measurement of milk yield in the lactating rat from pup weight and weight gain, *J. Pead. Gastroenterol. Nutr.*, **3,** 613-617.

Sandow, B.A., West, N.B., Norman, R.L., & Brenner, R.M. (1979). Hormonal control of apoptosis in hamster uterine luminal epithelium, *Am. J. Anat.*, **156**, 15.

Rosl, F. (1992). A simple and rapid method for detection of apoptosis in human cells, *Nucleic Acids Res.*, **20,** 5243.

Saskela, O. & Rifkin, D.B. (1988). Cell-associated plasminogen activation: regulation and physiological functions, *Ann. Rev. Cell. Biol.*, **4,** 93-126.

Savill, J.S., Henson, P.M. & Haslett, C. (1989). Phagocytosis of aged human neutrophils by macrophages is medaited by a novel "charge sensitive" recognition mechanism, *J.Clin. Invest.*, **84,** 1518-1527.

Savill, J.S., Dransfield, I., Hogg, N. & Haslett, C. (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis, *Nature*, **343**, 170-173.

Savill, J., Fadok, P., Henson, P. & Haslett, C. (1993). Phagocyte recognition of cells undergoing apoptosis, *Immunol. Today*, **131**, 14.

Schanne, F.A., Kane, A.B., Young, E.E & Faber, J.L. (1979). Calcium dependence of toxic cell death: a final common pathway, *Science,* **206,** 700-702.

Schmidhauser, C., Bissell, M. J., Myers, C.A. & Casperson, G.F. (1990). Extracellular matrix and hormones transcriptionally regulate bovine beta-casein 5' sequences in stably transfected mouse mammary cells, *Proc. Natl. Acad. Sci. USA,* **91,** 9118-9122.

Schmidhauser, C., Myers, C.A., Mossi, R., Casperson, G.F. & Bissell, M.J. (1995). Extracellular matrix dependent gene regulation in mammary epithelial cells, In *Intracellular signalling in the mammary gland,* (eds. Wilde, C.J., Peaker, M. & Knight, C.H.), 107-120, Plenum Press, London.

Schneider, C., King, R.M. & Phillipson, L. (1988). Genes specifically expressed at growth arrest of mammalian cells, *Cell,* **54,** 787-793

Schwartzman, R.A. & Cidlowski, J.A. (1993). Apoptosis: The biochemistry and molecular biology of programmed cell death, *Endocrine Reviews*, **14**, 133-151.

Seely, K.A. & Aggeler, J. (1991). Modulation of milk protein synthesis through alteration of the cytoskeleton in mouse mammary epithelial cells cultured on a reconstituted basement membrane, *J. Cell Physiol.*, **146,** 117-130.

Selvakumaran, M., Lin, H.K., Miyashita, T., Wang, H.G., Krajewski, S., Reed, J.C., Haffman, B. & Liebermann, D. (1994). Immediate early up regulation of bax expession by p53 but not TGFß1: A paradigm for distinct apoptotic pathways, *Oncogene*, **9**, 1791-1798.

Sell, C., Baserga, R. & Rubin, R. (1995). Insulin-like growth factor 1 (IGF-1) and IGF-1 receptor prevent etoposide-induced apoptosis. *Cancer Res.*, **55,** 303-306.

Sensibar, J.A., Liu, X., Patai, B., Alger, B & Lee, C. (1990). Characterisation of castration induced cell death in the rat prostate by immunohistochemical localisation of cathepsin D, *Prostate,* **16,** 263-271.

Sheffield, L.G., & Kotolski, L.C. (1992). Prolactin inhibits programmed cell death during mammary gland involution, *FASEB J.*, **6,** A11843.

Shipman, L.J., Docherty, A.H., Knight, C.J. & Wilde, C.J. (1986). A study in vivo of mammary differentiation and involution during a normal lactation cycle in the mouse, *Biochem. Soc. Trans.*, **615,** 443-444.

Shipman, L.J., Docherty, A.H., Knight, C.H. & Wilde, C.J. (1987). Metabolic adaptation in mouse mammary gland during a normal lactation cycle and in extended lactation, *Q. J. Exp. Physiol.*, **72,** 303-311.

Sinah, Y.N. & Tucker, H.A. (1966). Mammary gland growth of rats between 10 and 100 days of age, *Am. J. Physiol.*, **210,** 601-605.

Stacey, A., Schnieke, A., Kerr, H., Scott, A., McKee, C., Cottingham, I., Binas, B., Wilde, C. & Colman, A. (1995). Lactation is disrupted by α lactalbumin deficiency and can be restored by human α lactalbumin gene replacement in mice, *Proc. Natl. Acad. Sci (USA),* **92,** 2835-2839.

Stacey, N.H., Bishop, C.J., Halliday, J.W., Halliday, W.J., Cooksley, W.G.E., Powell, L.W. & Kerr, J.F.R. (1985). Apoptosis as the mode of cell death in antibody dependent lymphocytotoxicity, *J. Cell, Sci.*, **74**, 169-179.

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

Streuli, C.H. & Bissell, M.J. (1990). Expression of extracellular matrix components is regulated by substratum, *J. Cell Biol,* **110**, 1405-1415.

Streuli, C.H., Bailey, N. & Bissell, M.J. (1991). Control of mammary epithelial differentiation: the separate roles of cell-substrata and cell-cell interactions, *J. Cell Biol.* **115,** 1383-1395.

Streuli, C.H., Schmidhauser, C., Kobrin, M. & Bissell, M.J. (1993). Extracellular matrix regulates expression of the TGF ß1 gene, *J. Cell Biol.*, **120,** 253-

Streuli, C.H., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, A.P.N., Roskelley, C. & Bissell, M.J. (1995). Laminin mediates tissue specific gene expression in mammary epithelia, *J. Cell Biol.* **129,** 591-603.

Streuli, C.H., Edwards, G.M., Delcommenne, M., Whitelaw, B.A., Burdon, T.G., Schindler, C. & Watson, C.J. (1995). Stat5 as a target for regulation by extracellular matrix, *J. Biol. Chem.*, **270,** 21639-21644.

Sulstun, J.E. & Horvitz, H.R. (1977). Post embryonic cell lineages of the nematode *Caenorhabditis elegans, Dev. Biol.*, **82**, 110-156.

Sympson, C.J., Talhouk, R.S., Alexander, C. M., Chin, S.K., Clift, S.M., Bissell, M. J. & Werb, Z. (1994). Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue specific gene expression, *J.Cell. Biol.*, **125,** 681-693.

Talhouk, R.S., Chin, J.R., Unemori, R.N., Werb, Z. & Bissell, M.J. (1991). Proteinases of the mammary gland: developmental regulation *in vivo* and vectorial secretion in culture, *Development,* **112,** 439-449.

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

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J.A. & Reed, J.C. (1995). Cloning and functional analysis of BAG-1: a novel Bcl-2 binding protein with anti-cell death activity, *Cell*, **80**, 279-284.

Tanaka, S., Saito, K. & Reed, J.C. (1993). Structure-function analysis of the bcl-2 oncoprotein, *J. Biol. Chem.*, **268**, 10920-10926.

Tenniswood, M., Guenette, R.S., Lakins, J., Mooribroek, M., Wong, P., & Welsh, J. (1992). Active cell death in hormone dependent tissues, *Cancer and Metastasis Reviews II*, 197-220.

Tenniswood, M., Taillefer, D., Lakins, J., Guenette, R.S., Mooibroek, M., Daehlin, L. & Welsh, J. (1994). Control of gene expression during apoptosis in hormone dependent tissues. In *Apoptosis II: The Molecular Basis of Cell Death*, (eds Tomei, L.D., & Cope, F.O.), Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Tonner, E., Beattie, J. & Flint, D.J. (1995). Production of an insulin-like growth factor binding protein (IGFBP), IGFBP-3 protease and expression of IGF-1 receptors by cells of the sheep immune system. *Eur. J. Endocrinol,* **132,** 118-122.

Topper, Y.J. & Freeman, C.S. (1980). Multiple hormonal interactions in the developmental biology of the mammary gland, *Physiol. Rev.*, **60,** 1049-1063.

Traurig, R.R. (1967). Cell proliferation in the mammary gland during late pregnancy and lactation, *Anat. Rec.*, **157**, 489.

Tremble, P., Damsky., C.H. & Werb, Z. (1995). Components of the nuclear signaling cascade that regulate collagenase gene expression in reponse to integrin-derived signals, *J. Cell Biol.*, **129,** 1707-1720.

Trivers, R.L.(1974). Parent offspring conflict, *Amer. Zool.* **34,** 249-264.

Truman, J.W. (1984). Cell death in invertibrate nervous systems, *Ann. Rev. Neurosci.*,

Tiong, T.S. & Herington, A.C. (1991). Tissue distribution, characterisation and regulation of messinger ribonucleic acid for growth hormone receptor and serum binding protein in the rat, *Endocrinology*, **129,** 1628-1634.

Tomei, L.D. (1991). Apoptosis; a programme for death or survival? In *Apoptosis; the molecular basis of cell death,* (eds. Tomei, L.D. & Cope, F.O.), 279-316, Cold Spring Harbour Laboratory Press, New York.
7, 171-188.

Trump, B.K., Berezesky, I.K. & Osornio-Vargas, A.R. (1981). Cell death and disease processes. The role of calcium. In *Cell Death in Biology and Pathology,* (eds. Bowen, I.D. & Lockshin, R.A.), 209, Chapman & Hall.

Trump, B.K., Berezesky, I.K., Sato, T., Laiho, K.U., Phelps, P.C. & De Claris, N., (1982). Cell calcium, cell injury and cell death, *Environ. Health Perspect.*, **57,** 281-294.

Tsujimoto, Y. & Croce, C.M. (1986). Analysis of the structure, transcripts and protein products of bcl-2, the gene involved in human follicular lymphoma, *Proc. Natl. Acad. Sci.*, **83**, 5214-5218.

Tucker, H.A. (1966). Regulation of mammary nucleic acid content by various suckling intensities, *Am. J. Physiol.*, **210,** 1209-1214.

Tucker, H.A. & Reece, R.P. (1963). Nucleic acid content of rat mammary glands during post-lactational involution, *Proc. Soc. Exp. Biol. Med.*, **112,** 1002-1004.

Ucker, D.S., Obermiller, P.S., Eckhart, W., Apgar, J.R., Berger, N.A. & Meyers, J. (1992). *Mol. Cell. Biol.*, **12**, 3060-3069.

Vaux, D.L., Cory, S. & Adams, J.M. (1988). Bcl-2 gene promotes haematopoietic cell survival and cooperates with c myc to immortalize pre B cells, *Nature*, **335**, 440-442.

Vaux, D.L., Weissman, I.L. & Kim S.K. (1992). Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2, *Science*, **258**, 1955-1957.

Vogelstein, & Kinzler, (1992). p53 function and disfunction, *Cell*, **70**, 523-526.

Vollmer, G., Michna, H., Schneider, M.R. & Knuppen,R. (1993). Stromal expression of tenascin is inversely correlated to epithelial differentiation of hormone dependent tissues, *Steroid Biochem. Mol. Biol.*, **48,** 487-494.

Walker, N.I., & Gobe, G.C. (1976). Cell death and proliferation during atrophy of the rat parotid gland induced by duct obstruction, *J. Pathol.*, **153**, 333-344.

Walker, N.I., Harmon, G.C., Gobe, G.C. & Kerr, J.F.R. (1988). Patterns of cell death, *Meth. Achiev. Exp. Pathol.*, **13,** 18-54.

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

Watt, F.M., Jordan, P.W. & O' Neill, C.H. (1988). Cell shape controls terminal differentiation of human epidermal keratinocytes, *Proc. Natl. Acad. Sci. USA*, **85,** 5576-5582.

Travers, M.T., Barber, M.C., Tonner, E., Quarrie, L.H., Wilde, C.J., Flint, D.J. (1996). The role of prolactin and growth hormone in the regulation of casein gene expression and mammary cell survival: relationships to milk synthesis and secretion, *Endocrinology,* **137,** 1530-1539.

Wang, Y., Szekely, L., Okan, I., Klein, G., Wiman, G. & Wiman K.L. (1993). Wild type p53 triggered apoptosis is detected by bcl-2 in a v-myc induced T cell lymphoma line, *Oncogene*, **8**, 3427-3431.

Waring, P. (1990). DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by riased inositol triphoshate levels, *J. Biol. Chem.*, **265**, 14476-14480.

Weedon, D. & Strutton, G. (1981). Apoptosis as the metabolism of involution of hair follicles in catagen transformation, *Acta. Derm. Venereol.*, **61**, 335.

Wellings, S.R. & Deome, K.B. (1963). Electron microscopy of milk secretion in the mammary gland of the C3H/Crgl mouse, Cytomorphology of the involuting gland, *JNCI*, **30**, 241-261.

Werb, Z., Banda, M.J. & Jones, P.A. (1980). Degradation of connective tissue matrices by macrophages: Proteolysis of elastin glycoproteins and collagen by proteinases isolated from macrophages, *J. Exp. Med.*, **152**, 1340-1457.

Whittemore, C.T. (1980). *In Lactation in the dairy cow.* London: Longman Press.

Wilde, C.J. & Kuhn, N.J. (1979). Lactose synthesis in the rat, and the effects of litter size and malnutrition, *Biochem J.*, **182**, 287-292.

Wilde, C.J., Henderson, A.J. & Knight, C.H. (1986). Metabolic adaptations in goat mammary tissue during pregnancy and lactation, *J. Reprod. and Fertility*, **76**, 289-298.

Wilde, C.J., Calvert, D.T., Daly, A. & Peaker, M. (1987). The effect of goat milk fraction on milk constituents by rabbit mammary explants and milk yield *in vivo*, *Biochem. J.*, **242**, 285-288.

Wilde, C.J., Henderson, A.J., Knight, C.J., Blatchford, D.R., Faulkner, A. & Vernon, R.G. (1987). Effects of long term thrice daily milking on mammary enzyme activity, cell proliferation and milk yield in the goat, *J. Animal Sci.*, **64**, 533-539.

Wilde, C.J., Addey, C.V.P., Casey, M.J., Blatchford, D.R. & Peaker, M. (1988). Feedback inhibition of lactation: The effect of a fraction on goat milk and composition, *Q. J. Exp. Physiol.*, **73**, 391-397.

Wilde, C.J., Blatchford, D.R., Knight, C.H. & Peaker. M. (1989). Metabolic adaptations in goat mammary tisue during long term incomplete milking, *J. Dairy Res.*, **56**, 7-15.

Wilde, C.J., & Knight, C.H. (1990). Milk yield and mammary function in goats during and after daily milking, *J. Dairy Res.*, **57**, 441-447.

Wilde, C.J., Blatchford, D.R. & Peaker, M. (1991). Regulation of mouse mammary

cell differentiation by extracellular milk protiens. *Exp. Physiol.*, **76,** 379-387.

Wilde, C.J. Addey, C.V.P., Boddy, L.M. & Peaker, M. (1995). Autocrine regulation of milk secterion by a protein in milk, *Biochem, J.*, **305,** 51-58.

Wilde, C.J., Addey, C.V.P., Boddy-Finch, L.M. & Peaker, M. (1995). Autocrine control of milk secretion, In *Intracellular signalling in the mammary gland,* (eds, Wilde, C.J., Knight, C.H. & Peaker, M.), 227-238, Plenum Press, London.

Williams, W.L., (1942). Normal and experimental mammary involution in the mouse as related to the inception and cessation of lactation, *Am. J. Anat.*, **71,** 1-41.

Williams, G.T., (1991). Programmed cell death: apoptosis and oncogenesis, *Cell,* **65,** 1097-1098.

Winder,S.J. & Forsyth, I.A. (1986). Insulin-like growth factor 1 IGF-1 is a potent miytogen for ovine mammary epithelial cells, *J.Endocrinol., supp., **108,*** 141.

Wu, X., Bayle, J.H., Olsen, D. & Levine, A.J. (1993). The p53-mdm-2 autoregulatory feedback loop, *Genes Dev.*, **7**, 1126-1132.

Wyllie, A.H., Kerr, J.F.R. & Currie, A.R. (1973). Cell death in the normal neonatal rat adrenal cortex, *J.Pathol.*, **111**, 255-261.

Wyllie, A.H., Kerr, J.F.R. & Currie, A.R. (1980). The significance of apoptosis, *Int. Rev. Cytol.*, **68**, 251-306.

Wyllie, A.H. (1980). Glucocorticoid induced thymocyte apoptosis as associated with endogenous endonuclease activation, *Nature*, **284**, 555-556.

Wyllie, A.H., Morris, R.G., Smith, A.L. & Dunlop, D. (1984). Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular systhesis, *J.Pathol.*, **142**, 67-77.

Wyllie, A.H., Morris, R.G. & Watt, A.E. (1986). Terminally differentiated and dying lymphiod cells contain nuclear endonuclease potentially responsible for chromatin changes in apoptosis, *J. Pathol.*, **148**, 94A.

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. & Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for bcl-xl and bcl-2, displaces bax and promotes cell death, *Cell*, **80**, 285-291.

Yonish-Rouach, E., Resnitsky, D., Lotem, J., Sachs, K., Kim-Chi, A. & Oren M., (1991). Wild type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin 6, *Nature,* **352,** 345-347.

Yuan, J. & Horvitz, H.R. (1990). The *Caenorhabditis elegans* genes ced-3 and ced-4

act autonomously to cause programmed cell death, *Dev. Biol.*, **138**, 33-41.

Yuan, J. & Horvitz, H.R. (1992). The *Caenorhabditis elegans* cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death, *Development*, **116**, 309-320.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. & Horvitz, H.R. (1993). The *C. elegens* cell death gene *ced 3* encodes a protein similar to mammalian interleukin-1ß converting enzyme, *Cell,* **75,** 641-652.

Zakeri, Z.F., Quagling, D., Latham, L., & Lockshin, R.A. (1993). Delayed internucleosomal DNA fragmentation in programmed cell death, *The FASEB J.*, **7**, 470-478.

Zacharchuk, C.M., Mercep, M., Chakraborti, P.K., Simons, S.S. & Ashwell, J.D. (1990). Programmed T lymphocyte death. Cell activation and steroid induced pathways are mutually antagonistic, *J. Immunol.*, **145**, 4037-4045.