The importance of cell division in udder development and lactation

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Abstract

The mammary secretory cell population increases in an exponential fashion during pregnancy in all species studied, as a consequence of very high rates of cell division. After parturition the mitotic index drops dramatically, but a limited amount of proliferation does continue, at least until the time of maximum milk yield. This is particularly true of rodents but also occurs in dairy species. During declining lactation apoptosis exceeds cell division, so the size of the cell population falls and it is this decrease which is responsible for the reduction in milk yield. Many factors influence cell division. In addition to well-known hormones such as ovarian steroids, prolactin and growth hormone, and growth factors such as epidermal growth factor and insulin-like growth factor-1, there are also effects of milking frequency and nutrition. Some of these same factors are now also known to regulate apoptosis. The challenge for the future is to understand more about the relationships between apoptosis and cell division in the mammary gland; for instance, are the two mutually exclusive and independent or is apoptosis important in preparing the gland for renewed cell division? To this end, we have developed a lactation rescue model which will allow us to study interactions between apoptosis and cell division in lactating mouse and cow mammary glands.

Keywords: Mammary gland; Development; Cell proliferation; Apoptosis; Lactation

1. Introduction

Growth of the mammary gland is termed mamogenesis. There are two distinct phases, growth prior to first conception and then cyclical waves of proliferation, secretion and involution during recurring lactation cycles. The most important part of the first phase is the allometric growth which occurs just before and around puberty, since inappropriate development at this stage can have long term repercussions (Sejrsen, 1994). Rapidly-reared heifers often have depressed milk yield, and research has shown this to be a consequence of low blood concentrations of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) reducing peripubertal mammary cell proliferation (Weber et al., 1999). The GH/IGF axis is not the only endocrine regulator of mammary proliferation, but it is an important element. During the life of the cow the mammary cell population probably expands by more than 100-fold (although precise quantification is difficult) with the vast majority of this proliferation taking place during pregnancy under the stimulatory influence of ovarian steroids and peptide mammogens, including GH. Treatment of late pregnant goats with exogenous GH
increases cell proliferation and subsequent milk yield (Knight et al., 1994), and there is much evidence to show that the number of secretory cells present in the mature udder is a primary determinant of milk yield (Sorensen et al., 1998). Using bromodeoxyuridine injections to “tag” proliferating cells and then counting these cells in histological sections obtained by biopsy, we have observed labelling indices of around 10% in late pregnancy, subsequently falling precipitously after parturition; mammary cells do not proliferate extensively during lactation, although they retain the potential to divide and simple treatments such as frequent milking are mitogenic (Wilde et al., 1987). GH administered in early lactation does not stimulate proliferation (Knight et al., 1994), but later in lactation its mitogenic capacity returns (Capuco and Byatt, 1998). Whether other mitogens are only active at certain stages of the lactation cycle is not known, but it is apparent that observations made at one point in time cannot be extrapolated to cover all circumstances.

2. Importance of cell number: comparative studies

A positive relationship between mammary gland size and milk yield has been recognised for many years (Linzell, 1966). This is easy to demonstrate in goats but less evident in cattle, due to the difficulty of obtaining reliable data on udder size. However, quick-setting polyurethane foam can be used to obtain casts of cow’s udders (Dewhurst et al., 1993), a technique which is relatively simple and which produces an accurate measure of gross udder size. Comparing a diverse group of cattle, we obtained a reasonable relationship between udder volume measured in this way and milk yield ($r = 0.704$, $P = 0.02$) but further calculations revealed that we were underestimating udder size in young heifers and overestimating it in old cows, due to the much more pendulous udders of the latter (Knight and Dewhurst, 1994). More recently, however, we have obtained much more convincing evidence of the above relationship, by comparing groups of high- and low-genetic merit (HGM, LGM) cows which were otherwise similar in age, body weight and stage of lactation. The HGM cows produced 1.3-times as much milk as the LGM cows, from 1.3-times as much udder tissue (Sorensen et al., 1998). Clearly, the amount of secretory tissue is an important determinant of milk yield, and selection for output has indeed resulted in larger udders.

Udder volume is a crude measurement of total tissue mass, and provides no information on the relative proportions of secretory and non-secretory tissue. Different tissue types can be distinguished by imaging techniques such as X-ray, computed tomography (CT) scanning and magnetic resonance imaging (MRI). We have used MRI in vivo in goats (Fowler et al., 1990) and others have used CT scanning in excised udders of heifers (Sorensen et al., 1987), but there is no possibility of using either method for dairy cows in vivo because cows are simply too big. The alternative approach is to measure the composition of the tissue by biochemical or histological analysis. We now do this in needle biopsy samples using a mechanical biopsy tool (Magnum; C.R. Bard, Covington, GA, USA) which allows for repeated measurements with relatively little disturbance to the cow. If it is the number of secretory cells which ultimately determines yield, one would anticipate that milk yield would correlate more strongly with udder DNA content (a measure of cell number) than with crude udder volume. A recent analysis shows that this is not necessarily the case; in a group of nine peak lactation cows we obtained a significant correlation between udder volume and milk yield ($r^2 = 0.63$, $P = 0.01$, Fig. 1a) but absolutely no relationship between total DNA and milk yield (Fig. 1b). The data set was small and it is also important to remember that an additional source of potential inaccuracy is introduced, since a single biopsy may not always be fully representative. Nevertheless, we have always maintained that milk yield is actually determined by the function of cell number and cell activity, and there was indeed evidence of greater enzyme activities (measured per cell) in higher yielding cows.

This all shows that cell number is an important determinant of peak milk yield, but it is not the only factor. However, in one respect, it would seem that cell number does become all-important, namely during declining lactation. Quantitative data for cattle is not yet available, but milk yield and cell number in goats decline in parallel after peak
DNA precursor, and proliferating cell nuclear antigen (PCNA), an endogenous proliferation-associated protein (McCormick and Hall, 1992). Mathematical approaches are also possible: a model of mammary growth was published recently which inferred rates of cell proliferation and cell death from changes in DNA content (Dijkstra et al., 1997). In order to arrive at the estimates, the authors assumed that cell death did not occur during pregnancy, that the rate of cell proliferation fell off exponentially after parturition and that the rate of cell death during lactation was constant. Figures are quoted for the rate of cell proliferation at parturition which range from 3% per day (goats) to 39% per day (guinea pigs). An inverse relationship between gestation length and the mammary growth exponent [it is an exponential process: (Knight and Peaker, 1982)] is inevitable, since the growth rate achieved in mice would, if replicated throughout gestation in dairy cows, produce an udder capable of filling several football stadia (Sheffield and Anderson, 1985), so it is perhaps not surprising that proliferation rate was lowest in goats. Unfortunately, however, the model also predicted considerable variation between different reports within the one species, from 10.8 to 33.4% in mice, for instance. Since one of these reports was from our own work (Knight and Peaker, 1982), I have calculated predicted mammary cell proliferation rates and our measurement of DNA content at day 12 of gestation. The lowest proliferation rate gave an accurate prediction (3.9 mg DNA vs. our measured value of 3.85 mg), while the other two overestimated size by up to 3.7-fold. It would be nice to think that the lowest proliferation rate was the other way around; our data indicated the highest rate, how critical is cell division as a determinator of cell prepartum DNA content. The reason is quite simple. Cell proliferation rate is a dynamic snapshot of what is actually occurring at the time, in this case on one particular day, whereas DNA content is a measure of growth that has gone on over a preceding period of time. In the case of the mammary gland this could be days, weeks or, in dairy species, even months. Proliferation can vary tremendously from day to day, for instance, doubling from day 1 to day 2 post-partum and then decreasing by almost 90% over the

![Graph](image)

Fig. 1. Relationship between milk yield and udder volume measured using polyurethane foam casting (a) or mammary DNA content determined in biopsy samples (b) for nine peak lactation cows. Relationship with udder volume was significant ($r^2 = 0.63$, $P = 0.01$), while the relationship with DNA content was not.

### 3. Measurement of cell division and apoptosis

Having established that cell number is important, how critical is cell division as a determinator of cell number, and how can we measure it reliably? Two specific phases of the cell cycle can be detected by histological methods, namely DNA synthesis (S phase) and mitosis per se. Mitotic index was replaced as the method of choice by the labelling index method obtained from tritiated thymidine incorporation into DNA, and this is in turn has largely been replaced by non-isotopic immunohistochemical detection methods such as bromodeoxyuridine, another DNA precursor, and proliferating cell nuclear antigen (PCNA), an endogenous proliferation-associated protein (McCormick and Hall, 1992).
next 2 days in the mouse (Knight and Peaker, 1982). Dijkstra et al. (1997) only quote a proliferation rate for one time point (parturition, a bad choice, given the extreme variability inherent around this time), although they do state that the rate varied during pregnancy. Since the highest parturient proliferation rate was associated with the lowest achieved size (based on our data set), one would assume that proliferation must previously have been much lower. The reverse was actually the case; we observed higher rates of cell proliferation at all other stages of pregnancy, and the model is almost certainly misappropriating the very high rates of proliferation which we observed postpartum. Mechanistically, this is a very misleading error and a good example of why “snapshot” data should not be inferred from mathematical modelling. But, it is also an excellent example of how very crucial cell proliferation around parturition is to mammary development. Because of the exponential nature of mammogenesis, a doubling of cell population occurring in the first few days postpartum (if it were to happen) would give a net increase in secretory cells of around 2.5-fold (mouse) or almost five-fold (goat) greater than if the same event happened at mid-gestation.

This all shows that snapshot measurements of mammary cell proliferation must be interpreted with caution. Exactly the same arguments can be applied to apoptosis, which is usually measured by detection of the fragmented DNA typical of non-necrotic cell death. The mathematical model assumed a constant rate of apoptosis during lactation which, in the case of the goat, was 0.4% of cells per day (Dijkstra et al., 1997). This seems a sensible figure. We have measured mammary DNA content at peak lactation in cows and obtained a figure of around 15 g (C.H. Knight, S. Robertson and A. Sorensen, unpublished observations), which would fall to about 5 g at drying-off at this rate of loss. This would equate well with our belief that cell number and milk yield decline in parallel; a fall in yield from 30 to 10 l would be very typical over this same time period. Unfortunately, the reality appears somewhat different. In the same peak lactation cows, we also measured rates of cell proliferation and apoptosis. The latter was less than 0.1% rather than around 0.4% and, more worrying still from the modelling standpoint, cell proliferation was approaching 20-fold higher than apoptosis (1.4% vs. 0.08%). If one assumed that these figures apply throughout lactation, then by drying-off time the cow’s udder would not have regressed at all, but would actually have more than doubled in size! Two things are apparent. Firstly, apoptosis is almost certainly not constant throughout lactation and cell proliferation continues throughout lactation at a slow (but significant) rate, so the model’s assumptions are inaccurate. Secondly, when using snapshot measurements, it is very important to know the “shutter speed”, i.e., the period of time over which the desired event is being detected. Consider the analogy of a photographer at a grand prix car race. If he takes a series of randomly-timed shots standing at a slow bend, his chances of getting good pictures of cars will be much greater than if he were to do the same thing half way down the fastest section of track. My (inaccurate) suggestion that the cow’s udder might grow rather than regress during declining lactation assumed that proliferation and apoptosis were being detected at the same point on the track, be it the hairpin or the straight, but we do not know that this is so. We used PCNA to measure proliferation and, in this case, the critical window is probably close to 1 day, since this endogenous protein is up-regulated during DNA synthesis with a half-life of around 20 h (Mc Cormick and Hall, 1992). Therefore, PCNA may give a good approximation of a daily proliferation rate. Apoptosis was detected using a commercial kit (Apoptag; Oncor, Gaithersburg, MD, USA) and, in this case, the fragmented DNA on which the method relies is only present for a few hours (Hande et al., 1998), meaning that the apparent rate may only be one-tenth (or thereabouts) of the daily rate. Even so, the observed rates in our peak lactation cows would still suggest a (small) day-on-day increase in mammary cell number rather than a decrease. We know that this does not happen, so the measurements made at peak lactation do not reflect later events.

For cell proliferation, but not yet for apoptosis, the problems caused by the snapshot approach can be overcome. It is possible to administer a DNA precursor (such as bromodeoxyuridine) repeatedly over a period of time, take a biopsy sample at the end of this period and thereby determine the sum of proliferation occurring over the whole of the period (assuming that bromodeoxyuridine-labelled cells do
not have a shortened life expectancy). As yet, very few data have been generated in this way.

4. Lactation rescue: a model system for investigating relationships between proliferation and apoptosis

For many years cell proliferation was intensively studied in the mammary gland and elsewhere, whereas for the last few years it has almost been forgotten in favour of apoptosis. However, it must be re-emphasised that changes in cell number result from the balance between the two processes (assuming necrotic cell death is minimal), and so it is very important to understand the relationship between the two. As we have just seen, simply recording rates of proliferation and apoptosis in normal mammary tissue can lead to erroneous results, so a model system is required in which the two events can be turned on and off such that their time courses and spatial relationships can be studied. The majority of apoptosis occurs during post-lactational involution, so a logical starting point would be to arrest lactation so as to turn on apoptosis and, having done so, attempt to reverse the process. If lactation were to restart, would it require renewed cell proliferation? We have done exactly this in mice (Sorensen and Knight, 1997, 1998).

The effects of removing pups from a day 6 lactating mouse, placing them with a foster mother and then replacing them with their own mother 48 h later are shown in Fig. 2. For the first day after replacement, weight gain was negative during the period when the pups were with their own mother (they spent 12 h with the foster mother to ensure they were fed). From the second day onwards the pups spent all their time with their own mother and did gain weight and, by the fourth day, their weight gain had recovered to normal levels. At this age (11 days) mouse pups are still entirely dependent on maternal milk, so it was evident that lactation had been successfully restored. Shorter and longer (up to 72 h) periods of separation resulted in faster and slower restoration, respectively, and lactation was also recoverable (but with greater difficulty) following separation later in lactation (Sorensen and Knight, 1997).

We then repeated the 48-h separation, but this time with administration of bromodeoxyuridine (to measure cell proliferation) during either the separation or the resuckling period. Apoptosis was measured using a commercial kit (Apoptag) as before. Compared with control mice with normal lactation, apoptosis was greatly increased during litter removal and then quickly returned to normal levels during resuckling (Table 1). This shows that the absence of a suckling stimulus results in involution and cell loss which can be reversed if the stimulus is reapplied. The highest level of bromodeoxyuridine incorporation was observed during the resuckling period, indicating that,

Table 1

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<thead>
<tr>
<th>Proliferation (%)</th>
<th>Apoptosis (%)</th>
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<tr>
<td>Control</td>
<td>4.60±0.53</td>
</tr>
<tr>
<td>Separated</td>
<td>9.50±0.81**</td>
</tr>
<tr>
<td>Resuckled</td>
<td>13.76±2.50*</td>
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</tbody>
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* Values are means±S.E. for groups of six mice.
** P < 0.05, *** P < 0.01, **** P < 0.001, all with respect to the respective control values.
not only was apoptosis turned off, but cell proliferation was also turned on in order to recover the lactation. This very neat picture is, however, incomplete. Whilst bromodeoxyuridine incorporation was highest during resuckling, it was also elevated above control levels during the separation period, i.e., when apoptosis was up-regulated and, we would have anticipated, proliferation was firmly turned off (Table 1). This raises the intriguing possibility that, faced with the absence of her pups, the lactating mouse decides to turn off lactation but does so in such a way that a fresh population of secretory cells will be readily recruitable should she have the need of them. Whilst it is possible that the same cue which starts apoptosis also starts a totally different series of events leading to cell proliferation, a more likely explanation is given in Fig. 3, which puts apoptosis into the context of the cell cycle. It may be that terminally-differentiated cells can enter directly into apoptosis, but there is also evidence to show that they do not do this, but rather that they re-enter the cell cycle, synthesise DNA (at which point they would incorporate bromodeoxyuridine) and then undergo apoptosis (Colombel et al., 1992). Assuming there is a “switch” at this point, the resumption of suckling would act to divert these cells away from the apoptotic path and keep them in a proliferative cell cycle. The “dual-signal” hypothesis of Evan and Littlewood (1993) envisages exactly this. They claim that apoptotic and proliferative pathways are intricably linked and that induction of apoptosis requires not only an initial signal causing cells to enter the cell cycle but also the subsequent absence of a survival signal which would otherwise “rescue” the cell. This reduces the likelihood of inappropriate proliferation (i.e., cancer) since two mutations would have to occur to achieve the desired combination of signals, but it also provides the necessary means by which mammary involution can be rapidly reversed.

5. Cell survival and lactation length

During the separation period, milk collected from the mice contained high levels of one particular IGF binding protein, IGFBP-5. This binding protein has previously been observed in involuting mammary tissue (Tonner et al., 1995) and was not present in milk from control or resuckled mice. IGF-1 is a known mammary mitogen and has been implicated as a mammary cell survival factor (see Flint and Knight, 1997), but its concentration is paradoxically elevated during post-lactational involution. In the presence of IGFBP-5, however, the cell survival function is lost. The post-lactational appearance of IGFBP-5 is prevented by administration of the suckling-related hormone, prolactin (Tonner et al., 1995), so it would appear that all the necessary elements of the dual-signal hypothesis are present. IGF-1 (possibly produced locally via GH stimulation of mammary endothelial cells) causes cells to enter the cell cycle and, if suckling occurs, prolactin is released, IGFBP-5 is down-regulated and the cell survival activity is turned on. When suckling is prevented, the cells lose their survival signal and apoptosis results, to be replaced by survival when suckling is restored (Fig. 3).

Although this model has been developed in rodents, there is no reason to believe that it does not also operate in dairy species, although it is important to recognise that the time course of events may be very different, since mammary involution is a much slower process in cows than in mice (Wilde et al., 1997). We are beginning to study lactation rescue in cows. The potential benefits of being able to unravel the mechanisms controlling cell proliferation and apoptosis in the lactating gland are immense. As I
stated earlier, apoptosis usually exceeds proliferation, so there is a gradual loss of secretory cells and a consequent reduction in milk yield. For this reason, dairy farmers have to rebreed their cows regularly and the cows have to cope with the dual metabolic burdens of lactation concurrent with gestation and the risks introduced by parturition. If we could move to a scenario where proliferation matched involution such that the population of secretory cells was stable, then milk yield should be sustainable at high levels and the cow would not need to be rebred so often (or even at all). The benefits of extended lactation have been considered previously (Knight, 1997).

6. Conclusions

Cell division is crucial to the mammary gland’s preparation for lactation. It is a major player in the determination of milk yield, but is not the only important factor; cellular differentiation also has a big influence. Cell lifetime must be taken into account, and it is lamentable that we still know very little about average life expectancy for individual mammary secretory cells. When considering changes in milk yield during the course of lactation and our ability to manipulate these changes, proliferation and apoptosis must be taken into account together and regarded as the whole process through which the cell population is modulated. I have argued for some time and continue to believe that it is during lactation itself that the greatest strides could be made towards more efficient and welfare-friendly dairying through an improved understanding of the control of both apoptosis and cell proliferation.

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