

Keratin 19 expression in the adult and developing human mammary gland

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Summary

In the adult human mammary gland, most of the luminal epithelial cells express keratin 19 (K19+). However, in some small ducts and terminal ductal lobular units where branching would be expected to occur during pregnancy, the pattern of expression of this keratin is heterogeneous. While the keratin 19 negative cells (K19-) appear to have a high proliferative potential *in vitro* and *in vivo*, they have a lower secretory activity than the K19+ cells as monitored by expression of secretory component in the resting breast or casein in the pregnant gland.

That the K19- cells form a separate proliferative compartment in the luminal cell lineage is suggested by the fact that they are absent in the prepubertal breast and only appear at puberty associated with branching ducts, and newly formed lobules. Our observations are consistent with the hypothesis that the K19- luminal cell is less differentiated than and may be precursor to the K19+ luminal cell, which represents the fully differentiated phenotype able to produce milk in response to a hormonal stimulus.

Introduction

The mammary gland is a tissue which undergoes major development after birth under the influence of hormones which effect growth and differentiation of the cells at puberty, pregnancy, lactation and involution. In the human, extensive proliferation of the parenchymal cells occurs at and after puberty resulting in the development of the mammary tree with extensive ductal branching and terminal ductal lobular units (TDLU, sometimes called lobules). Further branching of ducts and ductules within the TDLU occurs in early pregnancy (Russo & Russo, 1987).

Two major epithelial cell phenotypes can be identified in the mammary gland, namely the basal cells, which can function as myoepithelial cells, and the luminal cells which acquire full secretory activity at lactation. However, cell lineages and precursor relationships between differentiation phenotypes within such lineages have only been partially defined even in the rodent (Dulbecco *et al.*, 1986). In the human, it is difficult to try to define cell lineages in a dynamic way, by showing a precursor relationship of one cell type

for another. It is possible, however, to define classes of cells using immunological markers, to monitor for the presence of these defined phenotypes at different stages of development of the gland, and to examine their proliferative potential in culture.

The epithelial keratins are proving to be extremely useful for defining the epithelial cell phenotypes in the mammary gland (Taylor-Papadimitriou & Lane, 1987). We have developed a range of antibodies which are monospecific for individual keratins. Using these antibodies, the luminal and basal cell phenotypes can be distinguished and subclassifications within these general classes are possible (Bartek *et al.*, 1985a; Nagle *et al.*, 1986). Of the two main epithelial cell phenotypes the proliferation and differentiation of the luminal phenotype is of particular interest, since the major cell type found in breast cancers exhibits features characteristic of this cell (Taylor-Papadimitriou *et al.*, 1983; 1989).

An examination of the expression of K19+ in the normal resting adult gland has shown that while most luminal cells expressed K19+, a subclass which does not express it can be detected in the smaller ducts and TDLU (Bartek *et al.*, 1985a). This subclass of cells shows a high proliferative potential in cultures of milk epithelial cells and it is found in increased numbers in benign hyperproliferative lesions and some *in situ*

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carcinomas (Bartek *et al.*, 1985a; Bartek *et al.*, 1986b; Gusterson *et al.*, 1987). These observations suggest that the K19+ luminal cells may form a separate proliferative compartment within the luminal cell lineage. It was, therefore, of great interest to examine the luminal cells in prepubertal and pubertal gland for keratin 19 expression to see whether there could be any association between the onset of growth of the parenchymal cells seen at puberty and the presence of K19+ luminal cells. We find that, while all luminal cells in the prepubertal breast are K19+, K19+ luminal cells do appear in the branching ducts and TDLU of the pubertal gland. Our observations provide further support for the idea that the K19+ luminal cells forms a specific proliferative compartment.

Materials and methods

Monoclonal antibodies

The development and characterization of antibodies BA16 and BA17 against human K19 has been described previously (Bartek *et al.*, 1985a). The third monoclonal antibody to K19, A53-B/A2 (Karsten *et al.*, 1985) was a generous gift from Dr U. Karsten (Central Institute for Molecular Biology, Berlin, GDR). Two monoclonal antibodies, SC-05 and MFG-12, recognizing different epitopes of human secretory component have recently been described in detail (Bartek *et al.*, 1989), as were antibodies DA7 and DC10 specific for the human keratin 18 (Lauerova *et al.*, 1988). The antibody HMFG-1 directed towards an epitope on a large molecular weight mucin is well characterized (Taylor-Papadimitriou *et al.*, 1981; Burchell *et al.*, 1983; Gendler *et al.*, 1988). All the above mentioned antibodies and the TF-1 anti-pig transferrin antibody (Bartek *et al.*, 1982) used as a negative control were applied as undiluted hybridoma tissue culture supernatants. The anti-smooth muscle actin monoclonal antibody was developed by Gabbiani (Skalli *et al.*, 1986; Gugliotta *et al.*, 1988).

Tissues

For analysis of normal adult breast tissue, we used either fresh surgical specimens from reduction mammoplasty operations or autopsy material obtained within 8–24 h of the time of death. Tissues representing developmental stages of normal mammary gland from birth to puberty (see Table 1) were autopsy samples (6–26 h post-mortem) obtained through the Department of Forensic Medicine and the 2nd Department of Pathology, Children's Hospital, J. E. Purkyne University, Brno. Small pieces of tissue were either soaked in Tissue-Tek II OCT compound (Miles, Wien, Austria) for 30 min and frozen in liquid nitrogen or fixed in methacarn (a mixture of methanol, chloroform and acetic acid, 6:3:1 by vol.) and embedded in paraffin.

Indirect immunoperoxidase staining

The indirect immunoperoxidase staining procedure, applied to both frozen and deparaffinized tissue sections, was essentially as described previously (Bartek *et al.*, 1985a) using peroxidase-conjugated rabbit antiserum to mouse immunoglobulins (Dako, Copenhagen, Denmark, diluted

1:50) as the second antibody, 3,3'-diaminobenzidine (Sigma, London, UK) as the chromogen and Haematoxylin to counterstain nuclei.

Immunoenzymatic double-staining

In double-immunolabelling studies, deparaffinized tissue sections were first incubated with one monoclonal antibody (usually either SC-05 or HMFG-1), and the reactivity was visualized with peroxidase-conjugate and diaminobenzidine as chromogen, resulting in a brown precipitate. After several washes with phosphate-buffered saline (PBS), the same sections were exposed to monoclonal antibody BA17 to K19. The reactivity with this antibody was then visualized using the B-Histogal-M staining kit (Farmitalia Carlo Erba, Milan, Italy) based on β -galactosidase-conjugated donkey anti-mouse immunoglobulin antiserum, and the nuclei were counterstained with carmine red.

Culture of epithelial organoids

The processing of tissue and separation of epithelial from stromal components were as described previously by Stampfer and colleagues (1980). Briefly, organoids were prepared by enzymatic digestion of reduction mammoplasty tissue by gentle rotation in collagenase and hyaluronidase. The organoids were then collected by filtration through controlled pore size polyester cloth and either cryopreserved or cultured immediately in multiple 35 mm dishes (Nunc, Denmark) in the serum-free medium MCDB 170 (Hammond *et al.*, 1984). For immunocytochemistry, the growing organoids were fixed directly in plastic dishes in a cold (-15°C) mixture of methanol and acetone (1:1 v/v) for 10 min, followed by standard indirect immunoperoxidase staining (Bartek *et al.*, 1985a).

Results

K19+ distribution in the adult resting gland

The distribution of K19+ in the basal and luminal cells lining the ducts and lobules of the mammary gland as deduced from immunohistochemical staining of tissue sections of adult normal gland is shown diagrammatically in Fig. 1. Luminal cells can be distinguished from basal cells using a variety of markers (Taylor-Papadimitriou *et al.*, 1989) among which are actin which is strongly expressed by basal cells (Fig. 2A), and keratin 18 which is only expressed by luminal cells throughout the gland (Lane 1982; Taylor-Papadimitriou & Lane, 1987; see Fig. 2A). Although K19+ has been found to be expressed mainly by simple epithelial cells lining glands or ducts, it can also be expressed heterogeneously in stratified epithelium (Bartek *et al.*, 1986b). In the breast, K19+ expression is predominantly luminal although this keratin can be expressed, albeit less strongly, by basal cells in the large duct and by clusters of cells in the basal layer of the nipple epidermis (see Fig. 3 and Table 1). In the smaller ducts and TDLU, however, K19+ expression is restricted to the luminal cells, and in 70–90% of these structures the luminal cells seen in sections

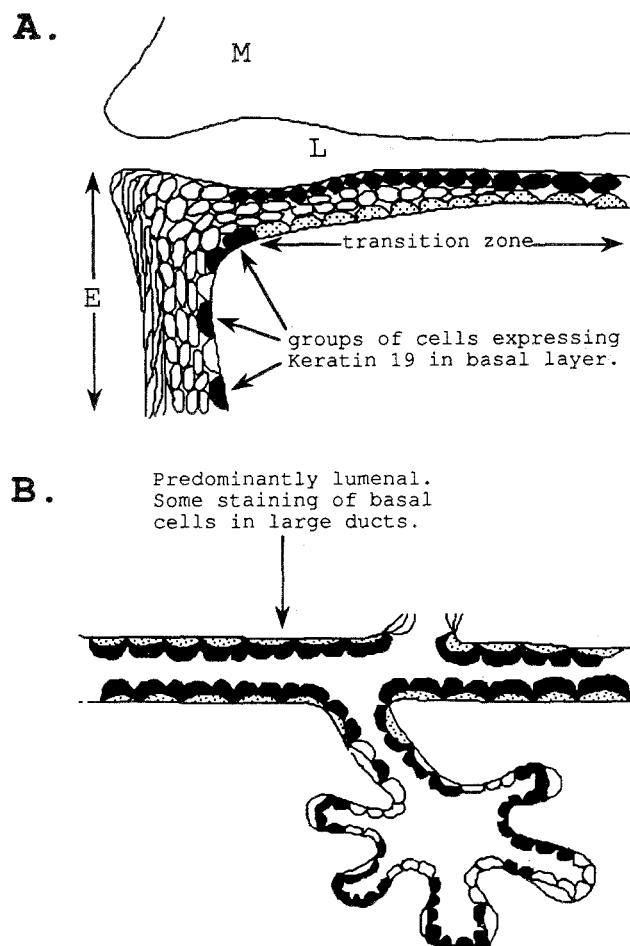


Fig. 1. Diagrammatic representation of K19+ expression in the human mammary gland. (A) The nipple epidermis and large ducts and (B) smaller ducts and TDLU. ■ Stain strongly with antibodies to K19+. ▨ Moderate or heterogeneous staining with antibodies to K19+. □ Unstained. M, Mesenchyme. L, Lumen. E, Epidermis.

show strong homogeneous staining. However, in some small ducts and TDLU, clusters of luminal cells can be found which do not express K19+. Most of the TDLU which contain K19- luminal cells show a mosaic pattern similar to that seen in Figs 2B and 2D. Very occasionally (less than 3% of all TDLU), all the luminal cells appear to be negative for K19+ expression.

The estimate of 10–30% for TDLU expressing K19+ in a mosaic fashion may be low, since a section will represent only a fraction of the total cells in the TDLU. That this might be so is suggested from observations on staining of primary cultures of whole organoids from reduction mammoplasty tissue with keratin antibodies. In medium MCDB 170 (Hammond *et al.*, 1984) the luminal phenotype does not proliferate but is visible as an upper layer of cells in the central core of the organoid, surrounded by proliferating cells of the basal phenotype (Taylor-Papadimitriou *et al.*, 1989). The luminal cells express keratin 18 homogeneously, but in more than 50% of organoids they show heterogeneity in expression of K19+ (see Fig. 4).

Association of K19+ expression with secretory function
Interestingly, the lack of K19+ expression in luminal cells appears to be related to decreased secretory function. We have previously shown that lobules containing a high proportion of K19- luminal cells do not secrete casein in the pregnant breast (Bartek *et al.*, 1987). We now find that in the adult resting breast, secretory component, which is involved in transport of IgA, is not expressed, or is expressed at a low level in TDLU containing many K19- luminal cells (Bartek *et al.*, 1987). However, a mucin which is produced in large quantities in milk (Shimizu & Yamauchi, 1982)

Table 1. Summary of K19+ expression in luminal cells in sections of human breast tissues during development.

Age Group	Number of cases (female/male)	K19+ staining pattern in luminal gland
Prepubertal		
Birth – 1 month	11 (5/6)	Homogeneously positive throughout the gland
1 month – 1 year	13 (8/5)	"
1 year – 10 years	9 (8/1)	"
Pubertal		
12 years – 17 years	7 (6/1)	>95% homogeneously positive. Rare single cells or clusters negative in TDLUs and 'budding' ducts of female breasts.
Postpubertal		
18 years and older	15 (15/0)	Large ducts and 70–90% small ducts and TDLU homogeneously positive. 10–30% TDLU heterogeneously positive with >50% luminal cells negative. <3% TDLU totally negative.

Tissues were fixed in methacarn and stained with antibodies BA17 and A53-B/A2

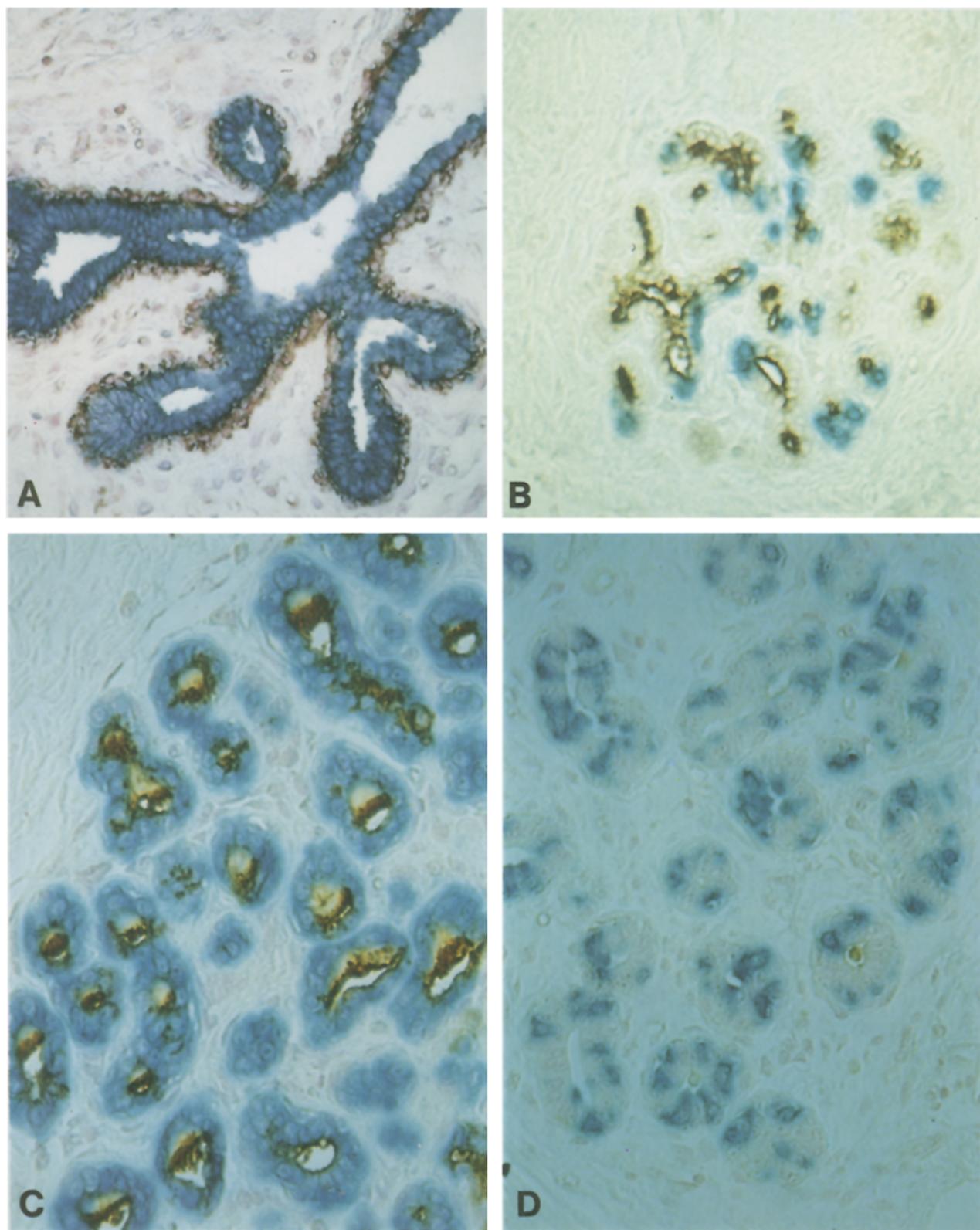


Fig. 2. Expression of various markers by breast epithelial cells and relation of keratin 19 expression to secretion of mucin and secretory component. (A) Basal cells in the normal resting breast show strong staining for actin (immunoperoxidase) while luminal cells are positive for keratin 18 (immuno- β -galactosidase). $\times 380$. (B) TDLU of the adult resting breast with heterogeneous (mosaic) pattern of K19+ expression (immuno- β -galactosidase reaction). Both K19+ and K19- luminal cells are able to secrete mucin as detected by immunoperoxidase staining with antibody HMFG1. $\times 240$. (C) Double-staining for K19+ (antibody BA17, β -galactosidase reaction) and secretory component (antibody SC-05, immunoperoxidase) demonstrates the most common TDLU phenotype characterized by homogeneous expression of K19+ and high levels of secretory component. $\times 380$. (D) TDLUs with heterogeneous expression of K19+ often lack production of secretory component completely or secrete only small amounts of this glycoprotein (double staining as in Fig. 2C). $\times 380$.



Fig. 3. K19+ cells in the basal layer of nipple epidermis. Some single cells or clusters of epithelial cells in the basal layer of nipple epidermis are strongly stained by the antibody BA17 (immunoperoxidase). $\times 300$.

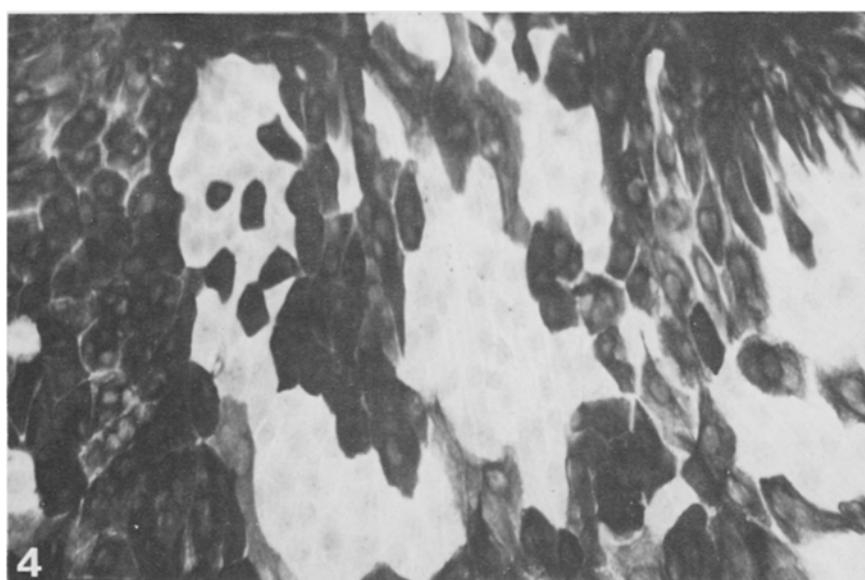


Fig. 4. Expression of K19 in cultured epithelium from reduction mammoplasty. Immunoperoxidase staining for K19 in primary culture of reduction mammoplasty organoids three weeks after plating reveals characteristic heterogeneity among cells in central areas of growing epithelial colonies (antibody BA16). $\times 200$.

and in lower amounts in the resting breast (Burchell *et al.*, 1983) appears to be expressed by both K19+ and K19- luminal cells (Fig. 2B). Figure 2 illustrates the high level of secretory component expressed by K19+ luminal cells (Fig. 2C) compared to the low expression by a TDLU containing predominantly K19- luminal cells (Fig. 2D). These observations suggest that the K19- luminal cell is a less differentiated phenotype than the K19+ luminal cell.

K19+ expression in the developing breast

The observations from immunohistochemical staining of sections and cultures of tissues from normal adult breast suggest that K19- luminal cells in the terminal structures of the gland may form a separate proliferative compartment. Since these structures begin to develop at puberty, from ducts existing in the pre-pubertal gland, we have examined the pattern of K19+ expression in the mammary gland from birth to

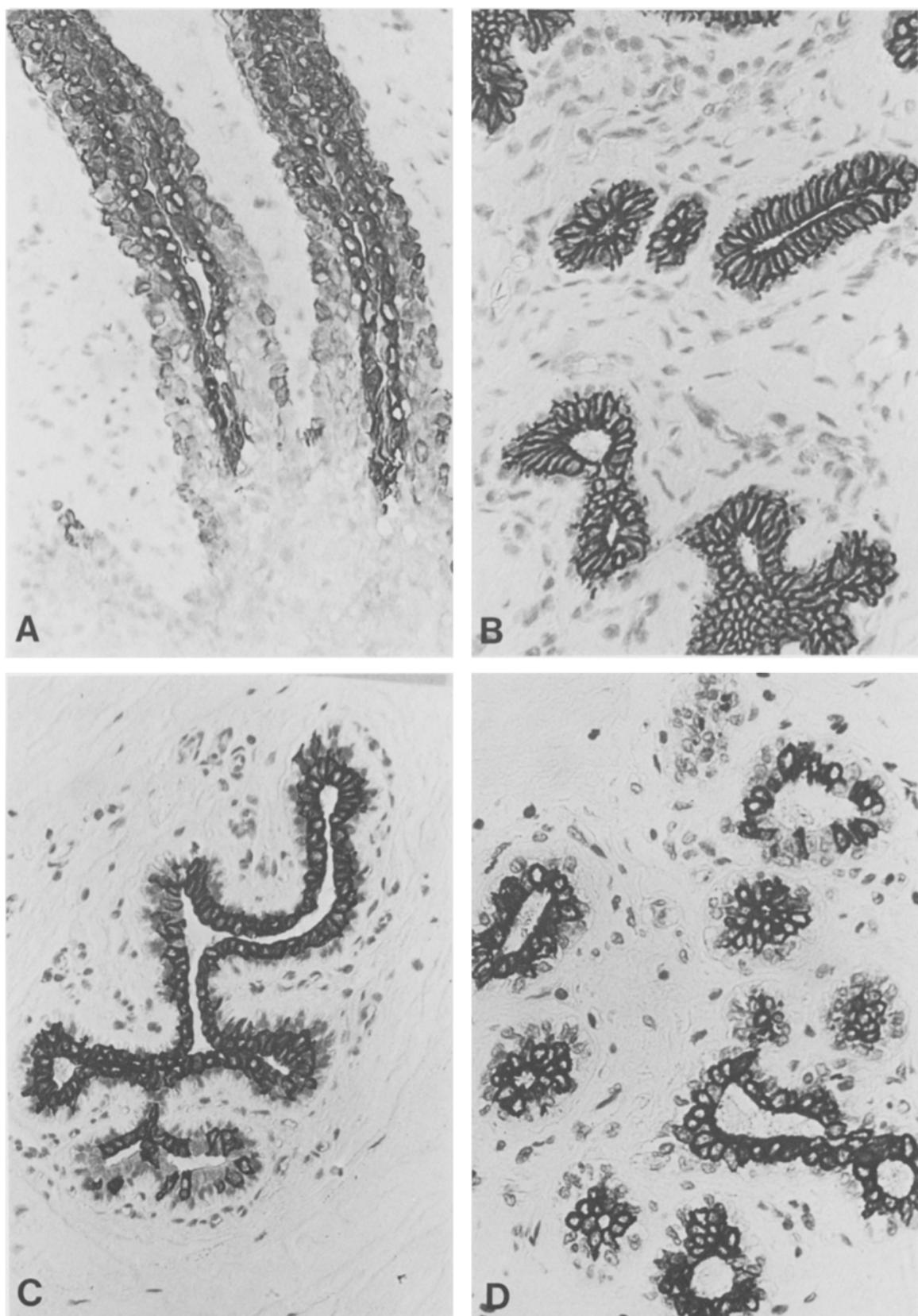


Fig. 5. Patterns of staining for K19 at various stages of human breast development. (A) Nipple epidermis with two large ducts of the female breast 25 days after birth. Strong positive staining for K19 is seen in rare cells in the basal layer of epidermis and in luminal ductal cells, while variable and weaker expression is also seen in basal cells of the large ducts in this subnipple 'transition' zone (immunoperoxidase with BA17). $\times 220$. (B) Homogeneous positivity of luminal cells and lack of K19 expression in basal (myoepithelial) cells of terminal epithelial structures six months after birth (immunoperoxidase, antibody BA17). $\times 350$. (C) Mosaic pattern of K19 $-$ and K19 $+$ luminal cells in a branching duct of the female breast at puberty (immunoperoxidase with antibody BA17). $\times 220$. (D) In newly formed TDLUs at puberty, some K19 $-$ cells can be detected besides the majority of K19 $+$ luminal cells (immunoperoxidase, antibody BA17). $\times 350$.

puberty, in sections of tissue taken at autopsy, using antibodies to detect the presence of the keratin. A striking observation is that, while in the prepubertal breast, all luminal cells throughout the gland show strong homogeneous staining with K19+ antibodies (Fig. 5B), at puberty, K19- luminal cells appear as single cells or cell clusters in the branching ducts (Fig. 5C and 5D, and Table 1). That the K19- cells in the luminal position are not displaced basal cells is shown by the fact that in serial sections they show positive staining with an antibody to keratin 18. As with the adult gland, K19+ cells are found in the basal layer of the nipple epidermis, in both the prepubertal and pubertal gland, and the keratin is detected in both luminal and basal cells in the large ducts (Fig. 5A). This 'transition' zone is proportionally shorter in the prepubertal gland.

Discussion

In the adult mammal, most of the tissues which proliferate extensively, such as the epidermis or the epithelium lining the gastrointestinal tract, show the same pattern of growth and differentiation throughout adult life. In contrast, the mammary gland, shows a dramatically different pattern in response to hormones at different stages of development. The extensive proliferation of the gland which occurs around puberty in the human female leads to morphogenesis, and at the cellular level this is manifest as a development of the ductal-lobular system which will form the basis of the functional structure involved in milk production. Identification of the stem or precursor cells which lead to the expansion of the epithelial cell population at puberty is of great interest, not only for understanding normal development, but also because these proliferating cells may be targets for the agents responsible for genetic changes which lead to breast cancer (McMahon *et al.*, 1970; McGregor *et al.*, 1979).

The identification of a subclass of luminal epithelial cells which do not express K19+ in the small ducts and TDLU of the adult gland, where branching might be expected to occur during pregnancy, led us to suggest that this luminal phenotype might form a separate proliferative compartment. The K19- phenotype was found to proliferate well in cultures of milk epithelial cells (Bartek *et al.*, 1985a) and was present in increased numbers in benign tumours (Bartek *et al.*, 1985b; 1986a), suggesting that this cell type did indeed have a high proliferative potential. The observation reported here that the K19- luminal cells appear around puberty in the branching ducts and TDLU, supports the idea that the phenotype represents a separate compartment which is seen in situations associated with proliferation in the normal gland.

Most of the luminal cells in the adult gland express K19+, and the proportion is highest during pregnancy

and lactation, i.e. when the gland is fully functional. Even in the resting gland, the K19+ cells produce large amounts of secretory component, suggesting that they are already committed to the secretory pathway. However, the K19- luminal cells in the resting breast either do not produce or produce smaller amounts of secretory component (this paper), and in the pregnant breast they do not produce casein (Bartek *et al.*, 1987). The K19- luminal cells, therefore, appear to be less differentiated than K19+ luminal cells, and it is possible that the latter develop from the progeny of the former.

A question which arises in view of the appearance of the K19- luminal cells at puberty is the identification of the cell from which they arise. Evidence from culturing human mammary epithelial cells from the adult gland suggests that they may develop from a K19- precursor cell in the basal layer (Taylor-Papadimitriou *et al.*, 1989). These could be the clear cells which can be defined by electron microscopy (Smith *et al.*, 1984), and which have been detected *in vivo* in the proliferating gland. It may be possible to establish more definitively a precursor relationship in the mammary cell lineages by introducing the β -galactosidase gene into the cells, and following the expression of the marker in K19- and K19+ cells in outgrowths in the nude mouse (Dubois *et al.*, 1987); attempts to carry out such experiments are underway.

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