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Original Scientific Article

CYTOKERATIN EXPRESSION IN MOUSE MAMMARY GLAND DURING FIRST FIVE WEEKS POST-PARTUM

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Summary: Changes during mammary gland development can be detected with methods using specific antibodies directed against specific cell structures. In the present study, the expression pattern of a type of intermediate filament called cytokeratins (CKs) was evaluated in tissue samples from mice mammary glands during the first five weeks post-partum (pp). Animals were divided into 5 homogeneous groups with 8 mice in each. Immunofluorescence and immunohistochemical staining procedures were used to determine various characteristics of different cells in the mammary gland. Several CKs were analyzed with specific markers and immunohistochemistry methods: CK5, CK7 and CK14 were detected in all weeks pp, although in different cell types; CK8 was positive in all periods except at week 1 pp; CK6, CK16 and CK19 were partially identified; CK1 and CK13 were not observed during the trial; and vimentin was detected in fibroblasts and fatty cells. It is known that CK expression varies with physiological and pathological changes, and it has been reported to mark different epithelial cell lineages; its evaluation is therefore of considerable importance for studies of breast cancer of a stem/progenitor cell origin, both in humans and animals. Our trial provides additional knowledge relative to the use of specific antibodies and techniques as valuable tools to detect CKs during early post-partum (pp) mice mammary gland development (weeks 1 to 5 pp), emphasizing the role of CKs as markers of mammary epithelial differentiation.

Key words: NMRI mouse; mammary gland; intermediate filaments; cytokeratin; vimentin

Introduction

The most resistant elements inside the cytoskeleton are the intermediate filaments. They form a cytoplasmic network, provide mechanical strength to cells, interact with other components of the cytoskeleton, and regulate protein localization and intracellular signaling (1). They are prominent in the cytoplasm of cells that are subjected to mechanical stress (2, 3), indicating that intermediate filaments are essential to cell growth and size because they regulate protein synthesis.

This multigene family is composed of more than sixty components, which can be subdivided into six categories classified into specific cell types according to their sequence homology, gene structure and assembly properties; the fifth category has its own characteristic features (4).

Cytokeratins (CKs), a set of polypeptides of different molecular weights, comprise the main type of intermediate filaments in epithelial cells and provide scaffold structures within cells (5). Immunohistochemical methods have been used to

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study the cellular expression and distribution of CKs, as well as to determine whether progression of the epithelium is accompanied by changes in these cytoskeletal structures. Furthermore, although murine strains do not show differences in CK expression, it is known that animal species can exhibit different CK polypeptides, and clear differences have been reported between CKs in mouse and rat hepatocytes, in contrast to epidermis CKs (6).

CK composition is extremely heterogeneous and depends on various factors such as level of differentiation or anatomical location. Little is known about intermediate filaments in the mammary gland, which is composed of four cellular types (7): myoepithelial cells, luminal alveolar cells, luminal ductal cells and basal cells. Mammary gland arises from the surface ectoderm during embryogenesis, relying on reciprocal epithelial-mesenchymal interactions for morphogenesis. Following birth, although mouse mammary gland grows isometrically with the body, it is rapidly expanded via branching during puberty from week 3 post-partum (pp) (8) and it develops the lactating function from week 5 pp (9) what culminates in a definitive mature gland at week 12 pp (8). Moreover, CK expression can be analyzed according to the different stages of mammary gland development and can be modified by the loss of normal tissue architecture, such as occurs in tumor progression and metastasis (10, 11), which can be useful as a diagnostic test using anti-CK antibodies (12, 13). In fact, gene expression studies have shown that basal-like breast tumors are associated with expression of basal-type CKs, such as CK5, CK6 or CK14 in humans (12, 14), and that CK8, CK18, CK19 and vimentin can alter their expression profiles during tumor development in animal models (5). Expression of specific CKs has been found to mark different epithelial cell lineages; therefore, their analysis can be particularly useful in studies of breast cancers of a stem/progenitor cell origin, both in humans and animals (8).

CK1 and CK10 are synthesized in the epidermis (15), CK3 and CK12 (16) as well as the CK complex 8/18 (17) in corneal tissue, CK4 and CK13 in the esophagus, CK5 and CK14 in the basal layer of stratified epithelia, CK6 in terminal end buds (18), CK7, CK8 and CK19 in simple epithelia, and CK13 in non-keratinized stratified epithelia (13). Due to morphological and biochemical changes in mammary glands, CK expression can be lost, but can be detected by immunohistochemistry in areas of myoepithelial proliferation, as well as enhanced expression of vimentin in proliferative areas with osseous or chondroid metaplasia (19). Immunohistochemical procedures using paraffin-embedded specimens are the method of choice to evaluate protein expression at a cellular level while preserving tissue architecture in normal and neoplasic tissues (20), and can be enhanced by immunofluorescence techniques in order to improve the identification of some structures (8).

In the present study, we used immunohistochemical and immunofluorescence methods to analyze and compare patterns of CK expression during mammary gland development from weeks 1 to 5 pp in experimental mice models.

Materials and methods

Animals and experimental design

The present study was carried out on 40 female Naval Medical Research Institute (NMRI) mice, selected because of their high reproductive capacity and low incidence of spontaneous mammary tumors before week 52 pp. NMRI mice were weighed one day before the beginning of the study and divided according to their average weight (20-40 g) into 5 homogeneous groups (weeks 1 to 5 pp) of 8 mice in each:, given that lactating mammary glands are developing from week 5 pp in accordance with Mínguez-González (9), and these weeks were established as different stages. Animals were housed in plastic cages measuring 180 cm^2 and maintained in a temperature controlled room (22-23ºC) on a twelve-hour light/dark cycle. Food and water were available ad libitum throughout the experiment. No clinical signs of parasitic or infectious diseases were observed, and faecal analyses (flotation, sedimentation and larval migration) were negative in all mice at the beginning of the tests.

Groups were sacrificed by cervical dislocation followed by immediate exsanguination. The study was carried out in accordance with the VICH guidelines for "Technical requirements for registration of veterinary medicinal products". The protocol of the experiment was approved by the ethics committee of the Faculty of Veterinary Sciences (León, Spain), where the trial was conducted.

Table 1: Cytokeratin (CK) and vimentin expression during weeks 1-5 post-partum (pp) in mouse mammary gland

ANTIBODY USED AND CYTOKERATIN REVEALED	WEEKS POST-PARTUM				
	1	2	3	4	5
Anti-CK5	++	++	++	++	++
LLO01 (CK14)	+	+	+	+	-
LP1K (CK7)	+	+	+	+	+
LP2K (CK19)	+/-	-	-	-	+/-
LLO20 (CK6)	-	-	-	-	+
Anti-CK6 (CK6)	-	-	-	-	+
LLO25 (CK16)	+/-	-	+/-	-	+
TROMA 1 (CK8)	-	++	++	++	++
Anti-CK1	-	-	-	-	-
Anti-CK13	-	-	-	-	-
Vimentin	+*	+*	+*	+*	+*

Key: (-) no stain; (+) positive stain in 10-50% cells; (++) positive stain in more than 50% cells; (+/-) variable result in the mammary tissue studied; (+*) positive stain in the mammary stroma.

Immunofluorescence technique

Left and right abdominal and thoracic glands were taken for this experiment. Samples from mammary glands were dissected and embedded in "Tissue-Tek ® CRYO-OCT" compound (Fisher Scientific, Spain), frozen in liquid nitrogen and cut into 5 μm-thin sections using a "Leitz 1720 Cryostat Microtome" (Wetzlar, Germany).

Frozen sections were subjected to indirect immunofluorescence in accordance with the protocol described by Mínguez-González (9). The expression of CK subtypes was evaluated using different antibodies on frozen tissue sections by means of indirect immunofluorescence (IFI). The following primary antibodies were used: anti-CK1 (rabbit, 1:500), anti-CK5 (rabbit, 1:500), anti-CK6 (rabbit, 1:500), LP1K anti-CK7 (mouse, 1:1), TROMA 1 anti-CK8 (rat, 1:4), anti-CK13 (rabbit, 1:500), LLO01 anti-CK14 (mouse, 1:1), LL025 anti-CK16 (mouse, 1:1) and LP2K anti-CK19 (mouse, 1:1) (Jackson ImmunoResearch Labs., USA), and clone LN-6 anti-vimentin (mouse, 1:100) (Sigma-Aldrich, Spain). Secondary incubation was performed according to the protocol described by Sun et al. (8), although Fluorescein-Isothiocyanate-Fluorochrome (FITC)-conjugated goat anti-rat, Texas-Red (TR)-conjugated anti-mouse and anti-rabbit secondary antibodies (Jackson ImmunoResearch Labs., USA) were used here. In addition, vimentin was examined using a streptavidin-biotin peroxidase complex commercial kit (Santa Cruz Biotechnology, Spain) on tissue embedded in paraffin wax.

The expression of CK subtypes was also evaluated by double-IFI. This technique was carried out to detect CK8 (TROMA 1 anti-CK8, rat, 1:2) with CK5 (anti-CK5, rabbit, 1:250) and CK6 (anti-CK6, rabbit, 1:250). The entire primary incubation was performed with TROMA 1/anti-CK5 or TROMA 1/anti-CK6, and the secondary incubation was performed with FITC-conjugated goat anti-rat for TROMA 1 and with TR-conjugated anti-rabbit for anti-CK5 and anti-CK6.

Slides were mounted in "Antifade medium" (Vector Labs., Cambridgeshire, UK), and images were taken using a "Leitz Diaplan Microscope" (Wetzlar, Germany) equipped with automatic fluorescein and rhodamine filter sets, with a "Wild MPS 51S" camera with a special "Kodakcolor VR 400S" film for immunofluorescence.

Immunohistochemical staining procedures

Left and right abdominal and thoracic glands were also taken for this method. Samples from these mammary glands were excised, fixed in 10% buffered formalin for at least 24 hours at

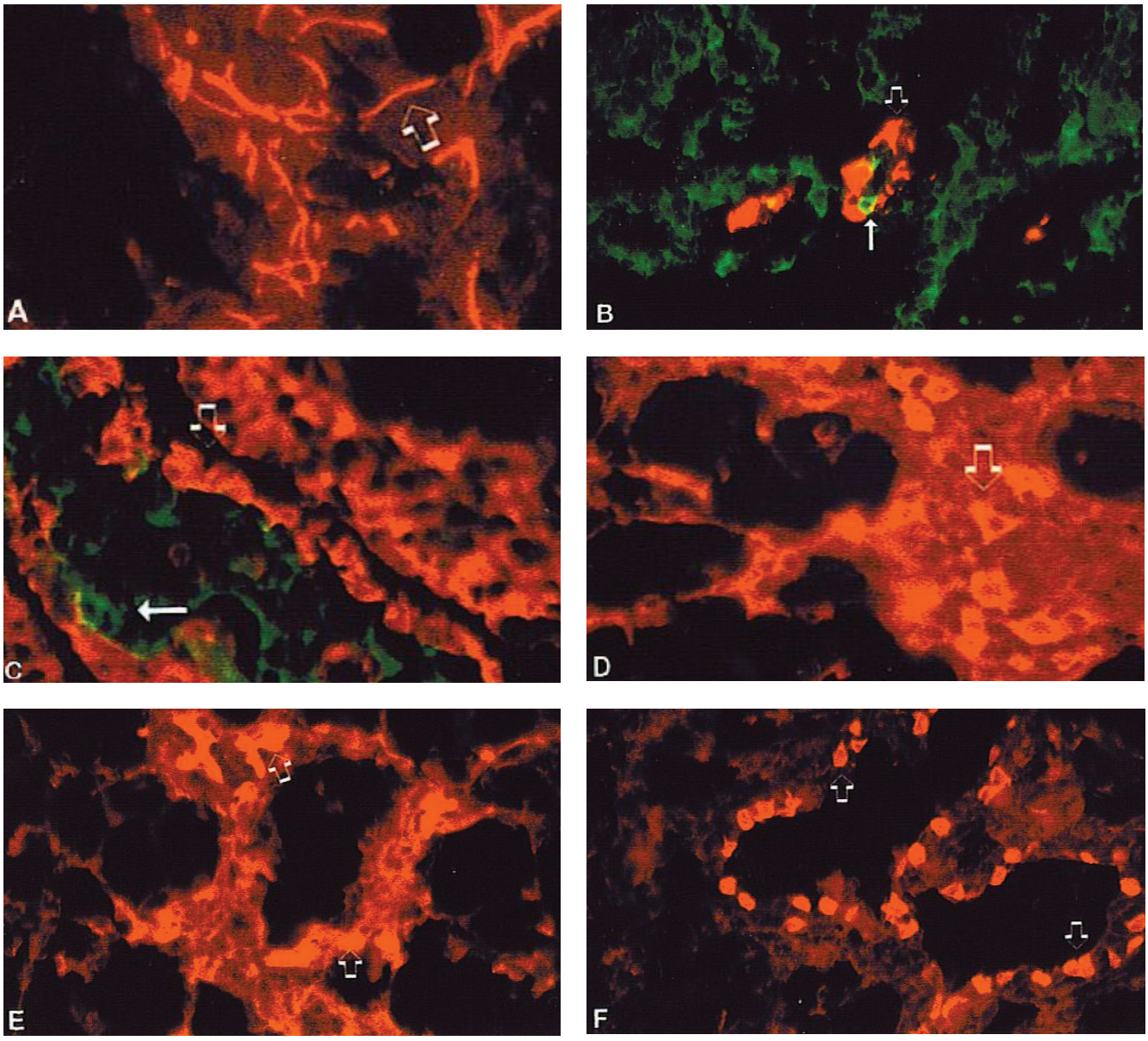

Figure 1: A. Expression of CK5 in mouse mammary gland during week 3 post-partum (pp). The thick arrow (⇒) indicates myoepithelial cells. IFI [anti-CK5 / 25x], **B.** Mammary tissue during week 3 pp. Luminal alveolar cells expressing CK8 are shown in green and indicated by a thin arrow (→). Myoepithelial cells around mammary alveoli expressing CK5 are shown in red and indicated by a thick arrow (⇒). Double-IFI [TROMA 1/anti-CK5 / 40x], **C.** Alveolus during week 3 pp. CK5 is shown in red and indicated by a thick arrow (⇒). CK8 is shown in green and indicated by a thin arrow (→). Double-IFI [TROMA 1/ anti-CK5 / 40x, **D.** CK7 expression in cells from lactating mammary glands, indicated by a thick arrow (⇒). IFI [LP1K / 40x], **E.** CK7 expression in scattered cells, indicated by a thick arrow (⇒). IFI [LP1K / 40x], **F.** Luminal ductal cells with CK7, indicated by a thick arrow (⇒). IFI [LPIK / 40x]

room temperature, embedded in paraffin wax, cut into 4 μm-thin sections using a "Leitz 1512 Rotatory Microtome" (Wetzlar, Germany) and examined for reactivity to the same panel of immunohistochemical markers as described for the immunofluorescence technique and using a streptavidin-biotin peroxidase complex commercial kit (Santa Cruz Biotechnology, Spain), in accordance with Rabanal and Else (21) and Mínguez-González (9), scoring the slides as negative (-) for no reactivity, (+) for weak reactivity, (++) for moderate reactivity and (+++) for strong reactivity (Table 1). Images were taken using an "Olympus AX70 Upright Compound Microscope" (Hamburg, Germany) with a "Multi Control Box U-MCB" equipped with "Kodakcolor VR 100S" film for immunohistochemistry.

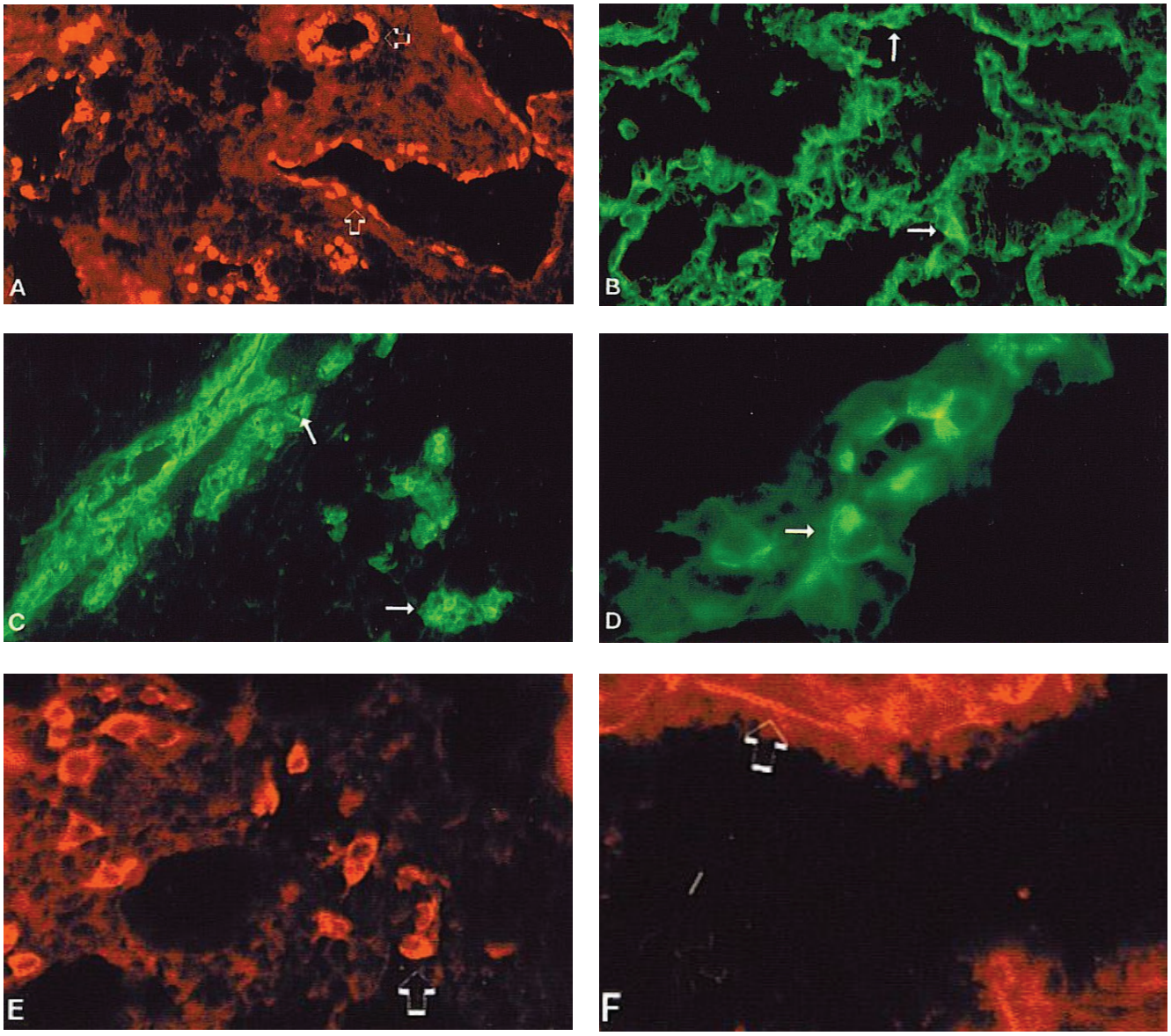

Figure 2: A. Tubular cells with CK7, indicated by a thick arrow (⇒). IFI [LP1K / 40x], **B.** Mammary gland during week 3 pp. CK8 expression in luminal alveolar cells, indicated by a thin arrow (⇒). IFI [TROMA 1 / 25x]. **C.** Mammary ducts stained with TROMA 1 antibody, indicated by thin arrow (→). IFI [TROMA 1 / 25x]. **D.** Mammary ducts stained with TROMA 1 antibody, indicated by a thin arrow (→). Intensity is more evident in the luminal segment of the cells. IFI [TROMA 1 / 100x], **E.** CK16 expression in isolated cells of a lactating mammary gland, indicated by a thick arrow (⇒). IFI [LL025 / 40x], **F.** CK14 expression in a mammary gland during week 1 pp. Myoepithelial cells surrounding mammary alveoli are indicated by a thick arrow (⇒). IFI [LLO01 / 40x]

Both immunofluorescence and immunohistochemical techniques were performed with negative controls where no primary antibody was added to the sections.

Results

CK5 was recognized by polyclonal antibody anti-CK5 and its expression pattern was similar from weeks 1 to 5 pp. Myoepithelial cells reacted with great intensity to the anti-CK5 and their characteristic cytoplasmic projections ("basket cells") surrounding the mammary alveoli could be observed (Figures 1A, 1B and 1C). Basal cells were positive in all samples (Table 2).

Regarding CK14, the LLO01 antibody produced a moderate reaction in myoepithelial (Figure 2F), luminal ductal and luminal alveolar cells, whereas basal ductal cells were negative in all the stages analyzed. During week 5 pp, only a few myoepithelial cells were detected (Table 2).

Table 2: CK expression in mouse mammary gland – Cellular types

CELLULAR TYPE				
Post-partum	Myoepithelial cells	Luminal alveolar cells	Luminal ductal cells	Basal cells
Week 1	CK5 and CK14	CK7, CK14 and CK16	CK7, CK14 and CK19	CK5
Week 2	CK5, CK7 and CK14	CK7, CK8 and CK14	CK7, CK8 and CK14	CK5
Week 3	CK5 and CK14	CK7, CK8, CK14 and CK16	CK7, CK8 and CK14	CK5
Week 4	CK5 and CK14	CK7, CK8 and CK14	CK7, CK8 and CK14	CK5
Week 5	CK5 and CK14	CK6, CK7, CK8 and CK16	CK7, CK8 and CK19	CK5

Some luminal alveolar and luminal ductal cells expressed CK7 in lactating mammary glands (Figure 1D). During week 5 pp, some isolated alveoli reacted positively to CK7 (Figure 1E) and only two mice showed this CK in every luminal ductal cell (Figures 1F and 2A). An increase was observed in the number of positive cells for CK7 as the stages progressed. LP1K antibody appeared in isolated myoepithelial cells during week 2 pp, as well as during weeks 1 to 5 pp in luminal alveolar and luminal ductal cells (Tables 1 and 2).

While CK19 was partially identified with LP2K during weeks 1 and 5 pp, CK6 was slightly positive with LLO20 and anti-CK6 during week 5 pp, and CK16 was detected with LL025 in some cells during weeks 1, 3 and 5 pp (Figure 2E) (Tables 1 and 2).

CK8 was recognized by the monoclonal antibody TROMA 1. There was a clear response, distributed in the same way in both luminal alveolar and luminal ductal cells (Figures 2B, 2C and 2D) in the mammary gland during weeks 2, 3 and 4 pp. There was an intense reactivity in luminal ductal cells in mammary samples from week 5 pp (Tables 1 and 2).

In contrast, CK1 and CK13 were negative in all stages, while vimentin was present in fibroblasts and fatty cells (Tables 1 and 2).

Discussion

Several CKs are lineage markers within the mammary epithelium (8) and can be analyzed by immunohistochemical and immunofluorescence procedures, providing relevant information on differentiation processes and cellular interaction dynamics (22). In histological sections it can be observed that the mouse mammary gland is mainly composed of adipose tissue with ducts and alveolar lobules scattered within it. Lobule-alveolar development reaches its peak during pregnancy and lactation; after these situations the mammary gland changes towards small ducts and terminal branches (23). Therefore, cellular differentiation implies obvious changes in stain, related to the pattern of CK expression, the nature of which is conserved throughout the mammary tree (20).

Luminal and basal cells express differentiation markers that gradually increase during mammary morphogenesis (24). During puberty and later, myoepithelial cells can be distinguished from luminal cells thanks to the expression of CK5. In the present study, myoepithelial cells expressed both CK5 and CK14 (typical for stratified epithelia) in all stages, and basal cells expressed CK5. These results agree with previous reports for mice (18, 25), dog (26) and human (27, 28) mammary glands. Furthermore, Sun et al. (8) detected CK5 expression in embryonic and prepubertal mammary glands. CK5 and CK14 usually appear in the basal layer of the epidermis (2). Moreover, the expression of these two CKs should be asynchronous during mammary gland development; this could be due to their link to other CKs through different binding proteins (29).

CK7, an important marker of ductal differentiation, was detected in all the stages analyzed, whereas CK19 expression was unclear. This finding is similar to those reported in previous experiments on human mammary glands (30, 31). It is known that CK19 is a typical marker of cholangiocytes with specific localization in the mammary gland, and it has been widely reported in cases of fibrosis provoked by helminthes such as *Fasciola hepatica* (32). An increase was observed in the number of cells positive for CK7

as lactation progressed, confirming that changes in cellular cytoskeleton are only detectable by immunohistochemistry, and not by conventional histology.

Our results showed that few cells expressed CK6 during week 5 pp, and CK16 was expressed during weeks 1, 3 and 5 pp. After using IFI in rat mammary samples, Lichtner et al. (7) failed to detect CK6, as did García et al. (33) and Mikaelian et al. (20) in mouse mammary glands; whereas Sapino et al. (18) identified CK6 in the mouse mammary tubular end buds. Using immunofluorescence, Smith et al. (34) detected the presence of CK6 in tubular end buds and in some cells from intralobular ducts, leading scientists to consider it as a marker (35, 36) for mammary pluripotent cells. CK6 is associated with epidermal proliferation and is expressed in epidermal hyperplasia in conjunctiva, oral mucosa and in some carcinomas (37, 38). In addition, Sun et al. (8) reported the presence of CK6 in most regions of embryonic and early postnatal mammary glands, and Grimm et al. (39) reported that the cross sections of nearly all embryonic mammary gland cells stained positive for this CK.

In contrast to the findings of previous studies on mice and rats (7, 40), we did not detect CK8 during week 1 pp. This may have been due to alterations in the cellular cytoskeleton structure as a result of physiological changes induced by hormonal status during week 1 pp, rendering CK8 inaccessible to the specific marker. Luminal cells have also been reported to be positive in other studies on human and animal models (8, 41, 42).

In contrast to humans, CK13 is not expressed in mouse mammary glands. Something comparable occurs with CK1 and CK10, which are only expressed in carcinogenic processes (43), particularly in transgenic mice and in human carcinomas (44). These results are similar to the negative detection of CK1, CK10 and CK13 at any developmental stage of the mouse mammary gland reported by Mikaelian et al. (20). Vimentin expression was similar to that found in the assay reported by Asch and Asch on mice (40) and others on dog (26) and human (45).

In conclusion, this report presents a comparative analysis of CK expression during the first five weeks pp. CK expression varies with physiological changes and tumor cells. Our study explored the use of specific CKs as markers of mammary epithelial differentiation in mice as an experimental model. By means of immunofluorescence techniques with specific antibodies for each CK, we detected several differences in the expression of these proteins among different cells from the same specimen. In addition, inter-species comparisons indicate similarities in relation to the pattern of CK expression.

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IZRAŽENOST CITOKERATINOV V MLEČNIH ŽLEZAH MIŠI V PRVIH PETIH TEDNIH PO KOTITVI

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Povzetek: Spremembe v mlečni žlezi med njenim razvojem lahko opazujemo z uporabo specifičnih protiteles proti posameznim celičnim strukturam. V opisani raziskavi smo ugotavljali izraženost vmesnih filamentov, imenovanih citokeratini (CK), v vzorcih tkiva mišje mlečne žleze pet tednov po kotitvi. Preiskovane miši so bile razdeljene v pet skupin po osem živali, v njihovih mlečnih žlezah pa smo s pomočjo imunofluorescenčne imunohistokemične metode ugotavljali značilnosti tkiva mlečne žleze. Ugotavljali smo izraženost različnih tipov CK. Prisotnost CK5, CK7 in CK14 smo zaznali pri vseh časovnih točkah, vendar pa so bili izraženi v različnih tipih celic. CK8 smo zaznali pri vseh časovnih točkah, razen prvi teden po kotitvi, CK6, CK16 in CK19 smo zaznali samo v nekaterih vzorcih tkiva, izraženosti CK1 in CK13 pa nismo zaznali v nobenem vzorcu. Poleg CK-jev smo v vseh preiskovanih vzorcih ugotovili tudi izraženost beljakovine vimentin, in sicer v maščobnih in vezivnih celicah (fibroblasti). Znano je, da je izraženost različnih CK-jev različna v različnih tkivih, da se lahko spreminja glede na fiziološko stanje tkiva in da so nekateri tipi CK-jev specifično izraženi v posameznih stopnjah razvoja epitelijskih celic. Poznavanje izraženosti posameznih tipov CK je zato pomembno za razumevanje razvoja posameznih celičnih linij epitelijskih celic mlečne žleze, ki lahko predstavljajo tudi izvor rakastih celic. Naša raziskava tako predstavlja prispevek o uporabi specifičnih protiteles kot metode za ugotavljanje izraženosti različnih tipov CK v času razvoja mlečne žleze pri miših po kotitvi (od prvega do petega tedna) in kaže na uporabnost različnih tipov CK kot označevalcev razvoja epitelijskhi celic mlečne žleze.

Ključne besede: miši NMRI; mlečna žleza; vmesni filamenti; citokeratin; vimentin