Tissue Culture of Feline Normal Mammary Gland

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ABSTRACT. To study the growth and morphology of feline mammary epithelial cells *in vitro*, normal feline mammary tissues as well as cells collected from feline milk were cultured and examined using phase-contrast microscopy and immunohistochemistry. The method employed was a modification of Hiratsuka *et al.* [8], and was found to be useful for the culture of dissociated feline mammary tissue. Small polygonal cells and large spindle-shaped cells that were positively stained with anti-keratin and anti-actin antibodies formed colonies with a pavement-like structure in culture of mid-pregnant mammary gland. Cultures of involuting mammary gland were composed mainly of small spindle-shaped cells, which were negative for keratin immunostaining and thought to be fibroblasts. Myofibroblasts, which participate in feline mammary carcinogenesis, and show keratin negative, actin positive, were not encountered in the present culture system of normal mammary gland. The cells from milk formed small colonies, but did not grow to reach confluent monolayer.—**KEY WORDS:** cat, culture, epithelium, immunohistochemistry, mammary gland.

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Recent advances in tissue culture methods have led to significant new discoveries in the fields of virology, cell biology and particularly of cancer research. Since the first description of a tissue culture study of human mammary carcinoma [3], there have been many investigations on the culture of mammary cells even in the veterinary field, including dogs [5, 13] and cattle [1, 4, 15]. Norval *et al.* [12] reported cell lines derived from feline mammary carcinoma. However, there has been little information about the cell morphology and growth characteristics in culture of feline normal and neoplastic mammary epithelial cells.

Since the feline mammary gland contains much stroma and adipose tissue, it is difficult to dissociate and obtain pure epithelial cells. Recently, Hiratsuka *et al.* [8] developed a simple method for the culture of human mammary epithelial cells using mechanical dissociation and enzyme digestion. This procedure is considered to be

applicable to the cultivation of feline mammary epithelial cells. The present work was carried out to investigate the proliferative properties and cellular morphology of feline normal mammary epithelial cells and also fibroblasts *in vitro* aiming at elucidation of cell biology of feline mammary tumor.

MATERIALS AND METHODS

Culture of dissected tissues from normal feline mammary gland: Samples of fresh mammary tissue, each about 1–3 cm³, were obtained from 3 cats in midpregnancy and 7 in involution by biopsy or autopsy. These tissues were stored in Eagle's minimum essential medium (E'MEM) (NISSUI 1, Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (29.2 mg/ml) for 1–2 h at 4°C for sterilization of tissue surface. Then each sample was dissociated by the method pre-

viously described by Hiratsuka *et al.* [8] with slight modification. Briefly, the tissue was finely minced with scissors and resuspended in digestion medium (E'MEM containing 360 U/ml collagenase Type I, Sigma Chemical Company, St. Louis, USA, and 10% FCS). Digestion was carried out in an atmosphere of 95% air-5% CO₂ at 37°C for 8–18 h. The cell suspension was then passed through a stainless steel mesh (mesh size; 80 µm) and the cells were collected by centrifugation at 1,000 rpm for 10 min. The supernatant was discarded. The pellet of sediment was resuspended by gentle pipetting in 10 ml of E'MEM, left to stand for 15 min, and the supernatant containing the lightest cells was cultured as the S-1 fraction. At the same time, the remaining pellet was resuspended by gentle pipetting in 5 ml of E'MEM and again left to stand for 10 min. The supernatant, which contained light cell groups, was cultured as the S-2 fraction. The pellet of sediment containing the rest of the cells was resuspended and cultured as the S-3 fraction.

Viable cells at a concentration of 5×10^5 /ml were incubated in a 50 mm dish containing several 18×18 mm² coverslips. The culture dishes were maintained in a humid 95% air-5% CO₂ atmosphere at 37°C. The medium was changed every 2–3 days and observed using a phase-contrast microscope (Nikon DIAPHOTO-TMD, Tokyo, Japan) every day.

When cultured cells formed a confluent monolayer, about 5–14 days after seeding, the cells were washed in 0.2 mM EDTA in phosphate-buffered saline (EDTA-PBS) and digested with 0.02% trypsin in EDTA-PBS by incubation at 37°C. Then E'MEM containing 10% FCS was added to stop the trypsinization. After washing of the cells, the number of viable cells was adjusted to 10^4 – 10^5 cells/ml. These cells were subcultured in a 50 mm dish containing several pieces of 18×18 mm² coverslips in the same

manner as that for the primary culture. At the same time, these cells were transferred to stock medium consisting of E'MEM containing 10% FCS and dimethyl sulfoxide, and stored at –80°C.

Culture of cells derived from feline milk: Samples of milk were obtained by manual expression from 10 lactating cats following oxytocin injection. The procedure for culture of cells from feline milk was essentially the same as that of Buehring [7]. These samples were centrifuged at low speed ($190 \times g$) and resuspended in E'MEM. The centrifugations were repeated 2 or 3 times in the same way. The cells collected were suspended in the culture medium and the number of living cells was counted. The culture was carried out in the same way as that of dissociated tissue described above.

Light Microscopy: Coverslips with attached cells were fixed in methacarn fluid and stained with hematoxylin and eosin (HE) or Giemsa stain.

Immunohistochemical Procedure: The coverslips with cultured cells were washed twice in PBS and placed in cold acetone (–20°C), then stored in a freezer for 30 min. Immunohistochemical staining was performed by the peroxidase-antiperoxidase procedure using rabbit anti-human keratin antiserum (Ortho Diagnostic Systems Inc., Cambridge, Massachusetts, USA), and rabbit anti-chicken actin antiserum (Miles Scientific Inc., Naperville, IL60566, USA), then each coverslip was processed with a sequence of solutions from a commercial kit (Ortho Diagnostic Systems Inc., New Jersey, USA) at room temperature. The reaction was developed using 3,3'-diaminobenzidine followed by counterstaining with hematoxylin.

RESULTS

I. Culture of mammary tissue from cats at midpregnancy and involution

Morphological findings by phase-contrast microscopy: In primary cultures of tissue from cats at both midpregnancy and involution, within 24 h after plating on glass dishes, the cells began to emerge from the attached organoids or cell clumps (Fig. 1) and proliferated. By 5–7 days in culture, cell groups of various shapes had formed on the coverslips. During the course of growth, these cell groups fused with each other and formed cell sheets. Spindle-shaped cells grew in all fractions, especially the S-1 fraction. In S-3, and occasionally the S-2 and S-1 fractions, the cells formed colonies with a typical pavement-like epithelial growth pattern consisting of polygonal cells on the glass dishes. Some of these colonies contained large spindle-shaped cells at their periphery. Small spindle-shaped cells were observed in spaces among the colonies of epithelial cells. Small spindle-shaped cells were predominantly proliferated in culture, especially the S-1 fraction and particularly from the tissue obtained during involution.

The first subcultures were done 1–2 weeks after plating. After the subculture, as the culture period progressed, polygonal cells showed a growth pattern similar to that in the primary culture, although the small spindle-shaped cells showed predominant proliferation. Multinucleated and degenerated cells in the epithelial colonies increased at every occasion of subculture. Finally, the epithelial cells ceased to grow and the small spindle-shaped cells grew over them. It was difficult to subculture the epithelial cells more than 3 transfers, i.e. for more than 30–40 days.

During the process of growth, lumen-like structures of various sizes were found in the center of small or large epithelial colonies in the S-2 fraction from midpregnant cats (Fig. 2).

Light microscopic findings: On HE-stained coverslips bearing the S-2 and S-3 fractions, colonies with a pavement-like

Fig. 1. In primary culture of tissue from cat, the cells begin to emerge from the attached organoid (O) within 24 h after plating on glass dish. Photographed by phase-contrast microscopy ($\times 100$).

appearance were seen. These consisted of polygonal cells attached to each other, with stronger HE staining of the cytoplasm than that of large and small spindle-shaped cells present at the periphery of the colony. A few vacuoles were also observed in the epithelial cell cytoplasm, and the nuclei of these cells were eccentric (Fig. 3).

Immunohistochemical findings: The small spindle-shaped cells showed no staining with anti-keratin antiserum. These cells were observed in all fractions and were considered to be fibroblasts. On the other hand, the polygonal cells forming pavement-like structures were stained strongly with the anti-keratin antibody. The large spindle-shaped cells present at the periphery of the colony or scattered among the small spindle-shaped cells showed weak staining for kera-

Fig. 2. Lumen-like structures of cultured cells obtained in midpregnancy, S-2 fraction. Photographed by phase-contrast microscopy ($\times 100$).

tin. According to these findings, the polygonal and large spindle-shaped cells were suggested to be epithelial elements. As for staining with anti-keratin antibody, no difference in cell components was observed between samples of midpregnancy and involution periods. The polygonal cells were stained strongly, mainly around the nucleus, by anti-keratin antiserum. On the other hand, the large spindle-shaped cells, though stained more weakly, revealed two staining patterns, one running parallel to the cell margin and the other forming a network throughout the whole cytoplasm (Fig. 4).

II. Cells derived from feline milk

Morphological findings by phase-contrast microscopy: By phase-contrast microscopy, though round cells floated in the medium, a few scattered single cells of various shapes, e.g., spindle-like or round, became attached within 1 or 2 days after plating. Some small

Fig. 3. Epithelial cells cultured from midpregnancy, S-3 fraction. A few vacuoles were also observed in the epithelial cell cytoplasm. HE ($\times 200$).

colonies were formed within 3 to 5 days in some instances. Such colonies were consisted of 5 to 20 polygonal cells. The cells contacted each other and some of them spread well on dish (Fig. 5). However, colony size did not increase thereafter. Finally the cells degenerated and exfoliated from the dishes. Therefore, observations of the cells on cover slip could not be performed.

DISCUSSION

The collagenase dissociation technique for the culture of mammary epithelial cells was first introduced by Lasfargues [9]. It was found in the present study that this procedure was also well suited to the culture of feline mammary epithelial cells. However, the cultured cells could not be subcultured

Fig. 4. Large, spindle-shaped keratin-positive cells in culture of mammary tissue obtained in the midpregnant period. They revealed two staining patterns, one running parallel to the cell margin and the other forming a network throughout the whole cytoplasm. PAP ($\times 400$).

more than 3 times or for more than 30–40 days, similar to the finding by Tateyama *et al.* [17], who studied canine mammary cell culture. A number of reports have described the stimulation of mammary epithelial cell growth *in vitro* by addition of prolactin [7], hydrocortisone [6], epidermal growth factor and steroid hormones [16] to the culture medium. In the present study, however, no hormones or promoting factors were added to the culture medium to facilitate study of the basic properties of feline mammary epithelial cells and fibroblasts. The limited growth capacity of normal mammary epithelial cells *in vitro* in conventional primary culture is a well-known phenomenon [10, 14]. Further studies are planned in order to study the effects of several growth factors including hormones.

Fig. 5. Cultured cells from feline milk forming a small colony. Photographed by phase-contrast microscopy ($\times 100$).

The typical growth pattern observed in the present study, i.e. polygonal cells in the center of each colony surrounded by large spindle-shaped cells, was also reported previously in canine mammary culture [17]. These features of cell growth seemed to somehow reflect the *in vivo* arrangement of the mammary gland. Since myoepithelial cells might be situated in the basement area, and the surrounding glandular epithelial cells in the organoids [11], the cell groups that emerged from the organoids possibly contained glandular epithelial cells and also myoepithelial cells [10]. Therefore, the polygonal cells and large spindle-shaped cells might be glandular epithelial cells and myoepithelial cells, respectively. This was confirmed by the immunohistochemical findings, which showed that the polygonal cells and large spindle-shaped cells growing in the pavement-like structures were kera-

tin-positive. Lumen-like structures were also observed in the S-2 or S-3 fraction in cultures of feline mammary cells, like those found in cultures of goat mammary cells derived from milk [18].

The method employed in the present study was modified from that of Hiratsuka *et al.* [8]. This could not clearly purify the epithelial cells from other cell components, as shown by the fact that epithelial cells were also present in the S-1 or S-2 fraction in some cases, whereas the S-3 fraction contained a considerable number of fibroblasts. This was thought to be due to the decrease in the number of fractions from 4 in the original method to 3 in the present one, which was necessary in order to examine both epithelial cells and fibroblasts in the same culture system. Observation of fibroblasts as well as epithelial cells in cultures of feline normal mammary cells is also necessary in order to study the role of these cells in the presence of feline mammary carcinoma, in which myofibroblasts play a significant role [19]. In the present study, the keratin-negative, small spindle-shaped cells were thought to be fibroblasts. However, we could not confirm presence of actin-rich, keratin-negative small spindle-shaped cells which might reflect the nature of myofibroblasts in culture of normal mammary gland exist. Such cell features might be detected more easily in cultures of mammary carcinoma tissue.

With regard to the cells obtained from feline milk, a few became attached to plastic dishes and formed small colonies. Adhesion of the cell to cell in such colonies seems to be constructing pavement-like structures and spreading on dish to show adequate cell activity. These observations indicate that mammary epithelial cells from feline milk can be cultured as same as the epithelial cells derived from goat milk [18]. Modification of the method such as use of hormones or other types of serum may improve the

culture of epithelial cell from feline milk.

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要 約

正常ネコ乳腺組織の組織培養：立山 晋・竹下明美・野坂 大・山口良二・三好宣彰・近藤房生¹⁾（宮崎大学家畜病理学教室，¹⁾同公衆衛生学教室）——乳腺上皮細胞の増殖状態とその細胞形態を研究するための一助として，正常ネコ乳腺組織およびその乳汁由来細胞の培養を試みた。乳腺組織からの上皮細胞の分離は平塚らの方法に準じて行い良好な結果を得た。増殖した細胞のうち多型細胞と大型紡錘型細胞は免疫組織化学的にケラチンおよびアクチンに陽性を示し，上皮細胞の特徴である敷石状の配列を示しながら増殖した。退行期の組織からはおもに小型紡錘型細胞が増殖し，これは抗ケラチン抗体に陰性であり，線維芽細胞と考えられた。ネコの乳腺腫瘍に特徴的に出現するケラチン抗体陰性，アクチン抗体陽性を示すと考えられる筋線維芽細胞は今回の正常乳腺の検索では観察できなかった。乳汁由来の細胞は培養皿に接着して小さいコロニーを形成したが，数日で剥離した。これは培養時の細胞数が少なかったためと考えられる。