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Isolation, purification, culture and characterisation of myoepithelial cells from normal and neoplastic canine mammary glands using a magnetic-activated cell sorting separation system

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ABSTRACT

Mammary gland tumours, the most common malignant neoplasm in bitches, often display myoepithelial (ME) cell proliferation. The aim of this study was to isolate, purify, culture and characterise ME cells from normal and neoplastic canine mammary glands. Monodispersed cells from three normal canine mammary glands and five canine mammary tumours were incubated with an anti-Thy1 antibody and isolated by magnetic-activated cell sorting (MACS). Cells isolated from two normal glands (cell lines CmME-N1 and CmME-N2) and four tumours (cell lines CmME-K1 from a complex carcinoma, CmME-K2 from a simple tubulopapillary carcinoma, and CmME-K3 and CmME-K4 from two carcinomas within benign tumours) were cultured in supplemented DMEM/F12 media for 40 days. Cell purity was >90%. Tumour-derived ME cell lines exhibited heterogeneous morphology, growth patterns and immunocytochemical expression of cytokeratins, whereas cell lines from normal glands retained their morphology and levels of cytokeratin expression during culture. Cell lines from normal glands and carcinomas within benign tumours grew more slowly than those from simple and complex carcinomas. This methodology has the potential to be used for in vitro analysis of the role of ME cells in the growth and progression of canine mammary tumours.

Introduction

In the normal mammary gland (NMG), the ductal and lobular system is lined by two cell layers, an inner or luminal layer and an outer layer composed of myoepithelial (ME) cells bordering the basal lamina. Mammary gland tumours are the most frequent malignant neoplasm in dogs and are known for their structural complexity and disputed histogenesis (Misdorp, 2002; Sorenmo, 2003). Malignant canine mammary tumours with ME cell proliferation (complex and mixed carcinomas) have a better prognosis than luminal-epithelial type simple carcinomas (Misdorp et al., 1999). In humans, ME cells play a role in cancer progression, as well as in the suppression of tumour growth and invasion (Sternlicht et al., 1997; Sternlicht and Barsky, 1997).

Isolation of enriched populations of epithelial and ME cells from mammary glands of several species, including rats, mice, rabbits, cattle and humans, has been achieved using density gradient centrifugation (Kraehenbuhl, 1977; McGrath et al., 1985; Zavizion et al., 1992), fluorescence-activated cell sorting (FACS; Sleeman et al., 2006; Keller et al., 2010; Rauner and Barash, 2012) and immunomagnetic cell separation methods (Clarke et al., 1994; Gomm et al., 1995). The latter include Dynabeads combined with ME cell-specific markers and magnetic-activated cell sorting (MACS), which have been used to separate human breast ME cells (Clarke et al., 1994; Gomm et al., 1995).

Thymocyte differentiation antigen 1 (Thy1, CD90) has been used as a marker for the isolation and/or in vitro identification of ME cells from NMGs in mice (Lennon et al., 1978; Kim and Clifton, 1993) and humans (Gudjonsson et al., 2002, 2005). Thy1 is an N-glycosylated glycophosphatidylinositol (GPI)-anchored conserved cell surface protein, originally identified as a thymocyte antigen (Barclay et al., 1976). In humans, Thy1 is expressed by fibroblasts, neurones and blood stem cells (Williams and Cagnon, 1982; Crawford and Barton, 1986; Craig et al., 1993; Saalbach et al., 1999). Expression of Thy1 by microvascular endothelial cells promotes invasion of malignant melanoma cells (Saalbach et al., 2002). Thy1 also plays a role in cell adhesion, proliferation and differentiation (Yamazaki et al., 2009).

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The aim of this study was to isolate, purify and culture ME cells from normal and neoplastic canine mammary glands through selection for Thy1 using MACS and to characterise the purified cells morphologically and immunophenotypically.

Materials and methods

Case selection

Five spontaneous mammary tumours and three NMGs were collected with the owner's permission from five bitches during surgery at the Department of Animal Pathology, University of Turin, Italy (Table 1). The three NMGs were from unaltered mammary glands in three of the bitches with tumours. Tissue samples from four tumours (cases 1, 2, 3 and 4) and two NMGs (cases 1 and 4) were used for ME cell isolation, purification, culture and characterisation, and for histological classification and immunophenotyping tumours, while tissue samples from one tumour and one NMG (case 5) were used for the same purposes, except that ME cells obtained were not cultured.

Histological classification and immunophenotyping of tumours

Tissue samples from tumours were routinely processed and stained for histological classification (Misdorp et al., 1999) and immunophenotyping using the avidin–biotin-peroxidase complex (ABC) method (Vector Laboratories) with monoclonal mouse anti-cytokeratin (CK) 5 antibody (clone PCK103; isotype IgG_1; Euro-Diagnostica; diluted 1:10), polyclonal rabbit anti-CK14 antibody (Covance Research; diluted 1:500) and monoclonal mouse anti-human calponin antibody (clone CALP; isotype IgG_1; Dako; diluted 1:400) (Vos et al., 1993a,b; Espinosa de los Monteros et al., 2002; Ramalho et al., 2006).

Immunohistochemical expression of Thy1

Fresh samples of NMGs and mammary tumours were frozen in liquid nitrogen and cryostat sections were prepared according to the method of Hellmén (1992). Blocks of tissue were covered with optimal cutting temperature (OCT) cryo-embedding media (Sakura) and stored at −70 °C. Cryostat sections (5 μm thickness) were cut at −20 °C and fixed in acetone at −20 °C for 10 min. Endogenous peroxidase activity was blocked by incubation with 0.05% phenyl-hydrazine (Sigma) in phosphate buffered saline (PBS; pH 7.2) for 40 min. Sections were covered with 10% normal rabbit serum in PBS for 30 min prior to incubation with monoclonal mouse anti-Thy1 antibody (clone 5E10; isotype IgG_1; BD Pharmingen; diluted 1:20) for 1 h at room temperature. The reaction was developed using EnVision (Dako), with 3,3-diaminobenzidine tetrahydrochloride (Sigma) as the chromogen, and nuclei were counterstained with Mayer's haematoxylin. The primary antibody was replaced with mouse IgG_1 (Dako) at the same dilution as a negative control.

Isolation, purification, culture and characterisation of myoepithelial cells

Preparation of monodispersed canine mammary cell suspensions - Fresh mammary tissue (approximately 0.5 cm3) was transported from the operating room on ice in Dulbecco's modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM/F12; Sigma–Aldrich) supplemented with 5% fetal calf serum (FCS), 5000 IU/mL penicillin and 5 mg/mL streptomycin. Tissue samples were transferred to glass Petri dishes and cut into small pieces (about 1 mm3). Disaggregated tissue was digested for 3.5 h at 37 °C under gentle rotation in DMEM/F12 supplemented with 300 U/mL collagenase and 100 U/mL hyaluronidase (StemCell Technologies). Following enzyme digestion, the supernatant containing the fat layer was decanted and the remaining organoids and single cells were transferred to centrifuge tubes, then the sample was centrifuged at 80 g for 30 s to remove blood vessels and fibroblasts. The supernatant was discarded and the cell pellet was washed three times with DMEM/F12 supplemented with 1% FCS.The remaining pellet was enriched for epithelial organoids according to the method of Stingl et al. (2001). A single cell suspension was obtained by sequential dissociation of fragments of mammary tissue by gentle pipetting for 1–3 min in 1–5 mL prewarmed trypsin–ethylene diamine tetraacetic acid (EDTA; Sigma–Aldrich). After addition of 10 mL cold Hank's Balanced Salt Solution (HBSS; Sigma–Aldrich) supplemented with 2% FCS, the suspension was centrifuged at 350 g for 5 min. The supernatant was removed and the pellet was treated with prewarmed 5 mg/mL dispase and 1 mg/mL DNase I (StemCell Technologies) for 1 min. The reaction was stopped in the same manner. The cell suspension was filtered through a 40 μm pore nylon mesh filter (BD Biosciences) to remove remaining cell aggregates and centrifuged at 350 g for 5 min, after which the supernatant was discarded. Viable cells were counted using a haemocytometer after staining with Trypan blue.

Isolation and purification of myoepithelial cells - Monodispersed cells were resuspended in MACS buffer containing PBS, 0.5% bovine serum albumin (BSA) and 2 mM EDTA, then incubated with the rat anti-canine Thy1 monoclonal antibody (clone YKIX337.217; isotype IgG_{2b}; AbD Serotec; diluted 1:125) for 30 min at 4 °C. Samples were washed by adding 1–2 mL MACS buffer per 1×10^7 cells and centrifuged at 300 *g* for 10 min.Magnetic beads conjugated to anti-rat IgG MicroBeads (Miltenyi Biotec) were incubated with the labelled monodispersed cells at 1×10^7 beads/mL for 15 min at 2–8 °C. Cells were washed with 2 mL buffer and centrifuged at 300 *g* for 10 min. The supernatants were aspirated and the pellets were resuspended in 500 μL buffer. Bead labelled ($Thy1^+$) cells were separated from unlabelled ($Thy1^-$) cells using a MACS Separator (Miltenyi et al., 1990). Viable cells were counted using a haemocytometer after staining with Trypan blue.

Culture of purified myoepithelial cells - Purified ME cells from NMGs 1 and 4 and mammary tumours 1–4 were cultured in tissue culture flasks, plates, dishes and uncoated chamber slides (Lab-Tek II Chamber Slide System, 8-well glass slide, Nalge Nunc). DMEM/F12 supplemented with 1% FCS, 2 mM glutamine, 1 μg/mL hydrocortisone, 5 μg/mL insulin, 10 ng/mL epidermal growth factor (EGF), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 50 ng/mL amphotericin B was added to all ME cell vessels (Gomm et al., 1997) and incubated at 37 °C in a 5% CO2 humidified incubator. Media were changed every 2–3 days (Gomm et al., 1995). At confluency, cells were washed with PBS, incubated with 0.25% trypsin–EDTA (Sigma–Aldrich) for 2–5 min (Hellmén, 1992) and reseeded at split ratios of 1:2–1:3 for 40 days. Cells were examined using an inverted phase contrast microscope (Nikon Eclipse TS1000).

Table 1
Clinical and pathological features of dogs with mammary tumours used for isolation of myoepithelial cells.

Case	Breed	Age (years)	Sex	Location of tumour	Size of tumour (cm)	Histological classification of tumour	Location of normal mammary gland
1	Hovawart	10	Female	IV right	4	Complex carcinoma	I right
2	Labrador retriever	10	Female	III right	1.5	Simple tubulopapillary carcinoma	–
3	Dachshund	10	Female	V right	2.3	Carcinoma within benign tumour	–
4	Yorkshire terrier	10	Female	II left	0.8	Carcinoma within benign tumour	V left
5	Mixed	9	Female	IV left	1.2	Complex carcinoma	I left

Table 2
Antibodies used in the immunocytochemical study.

Antigen	Antibody type	Clone	Isotype	Dilution	Source	Cases in which the antibody was used
Thy1	Monoclonal	YKIK337.217	IgG_{2b}	1:50	AbD Serotec	1, 2, 3, 4, 5
CK5	Monoclonal	PCK103	IgG_1	1:10	Euro-Diagnostica	1, 2, 3, 4
CK14	Polyclonal	–	–	1:500	Covance Research	1, 2, 3, 4, 5
CK19	Monoclonal	RCK108	IgG_1	1:100	Dako	1, 2, 3, 4
Vimentin	Monoclonal	V9	IgG_1	1:50	Dako	1, 2, 3, 4
SMA	Monoclonal	HHF35	IgG_1	1:100	Dako	1, 2, 3, 4
Calponin	Monoclonal	CALP	IgG_1	1:400	Dako	1, 2, 3, 4

Thy1, thymocyte differentiation antigen 1; CK, cytokeratin; SMA, smooth muscle α-actin.