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Cyclin D3 Expression in Normal Fetal, Normal Adult and Neoplastic Feline Tissue

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Summary

Cyclin D3 is a tightly regulated cell cycle protein and member of the cyclin D family—a group of proteins that facilitates the progression of a cell through G_1 and into the S phase of the cell cycle. All cells use at least one of the cyclin D proteins for cell cycle regulation. In this study, feline tissues (normal fetal and adult, and neoplastic) were examined immunohistochemically for expression and topographical distribution of cyclin D3. Its distribution was similar to that in human tissues in health and neoplasia, and suggested a dual role of cyclin D3 in cell proliferation and differentiation. Immature lymphoid tissue and proliferating epithelial cells in health and neoplasia were immunoreactive for cyclin D3, whereas expression of the protein in other immunoreactive tissues reflected differentiated cell types. Immunoreactivity for cyclin D3 was particularly striking in germinal centre cells of normal lymph nodes and B-cell lymphomas, and in normal suprabasal epithelial cells of the skin and mucous membranes of the oropharynx and in squamous cell carcinomas at these sites.

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Introduction

The cell cycle consists of four distinct phases, separated by "checkpoints". The S, G_2 and M phases are generally consistent and uniform in duration, and the rate of cell division is governed primarily by the G_1 phase (Andreeff *et al.*, 2000). The coordinated events of the cell cycle are regulated by the activity of cyclins, their catalytic cyclin-dependent kinases, and their inhibitors. Cyclin-dependent kinases form a group of highly conserved regulatory enzymes that are of critical importance in enabling a cell to proceed into its next phase of the cell cycle (Harper, 1997). There are multiple subsets of cyclin-dependent kinases, of which subsets cdk2, cdk4 and cdk5 require a conserved group of regulatory proteins known as the cyclins to bind and stimulate forward cell cycle progression. The cdk/cyclin complex has a proregulatory role in the cell cycle, but two families of inhibitory proteins can act at the same level of the cdk/cyclin complex and restrict the cell from dividing. These inhibitory proteins include kinase inhibitory proteins (KIPs) and the inhibitory kinases (INKs) (Reed, 1997; Kato, 1999; Sherr and Roberts, 1999).

Aberrant cell cycle regulation is characteristic of neoplastic cells—cells that continue to proliferate in the absence of stimulatory signals. Neoplastic cells often have a higher growth fraction than normal cells and major dysregulation of the cell cycle occurs at the checkpoints (Reed, 1997; Kato, 1999; Andreeff *et al.*, 2000). Methods and reagents are now available to facilitate the study of individual components of the cell cycle. The cyclin D family contains three isoforms—D1, D2 and D3. Cyclin D complexes with cdk 4 or 6 and acts to regulate the cell cycle at G_1/S phase. The role of cyclin D

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expression in both normal and neoplastic human tissue has been explored (Doglioni *et al.*, 1998; Teramoto *et al.*, 1999; Moller *et al.*, 2001). The cyclin D3 isoform facilitates physiological progression from G_1 to S after mitogenic stimulation in some cells, whereas its expression in other cells appears to be related to cellular differentiation. Dysregulation of cyclin D3 contributes to the high growth fraction of some murine and human tumours, but little is known of its role in tumours of veterinary importance.

In the present study, cyclin D3 expression was examined immunohistochemically in normal feline tissue and in a variety of feline neoplasms.

Materials and Methods

Tissues

Paraffin wax blocks selected from the pathology archive of the University of California Veterinary Medical Teaching Hospital (1987–2002) included an entire midgestation feline fetus, normal feline tissue and various feline tumours. Neoplastic tissues selected were oral squamous cell carcinoma ($n=22$), cutaneous squamous cell carcinoma (16), basal cell tumour (23), lymphoma (33), intestinal or colonic adenocarcinoma (13), mast cell tumour (5), thyroid adenoma and carcinoma (5) and meningioma (5). Normal feline tissues included stomach (2), intestine (2), oral/pharyngeal mucosa and larynx (2), lymph node (4), pancreas (2), thyroid gland (2), skeletal muscle (2), tongue (2), skin (4), urinary bladder (2) and single specimens of lung, uterus, ovary, liver, salivary gland, gall bladder, spleen, kidney, mammary gland and adrenal gland. Hyperplastic lymph nodes (2) and lymphoid depleted lymph nodes (3) were also examined. For each specimen, sections (5 μm) were stained with haematoxylin and eosin (HE) and examined by light microscopy. Tumours were first examined for confirmation of previous histological classification. The tissue samples selected had been fixed in formalin for no more than a limited period (<48 h) to prevent protein denaturation that might otherwise have interfered with appropriate immunohistochemical labelling.

Because preliminary immunolabelling showed particularly strong reactivity in skin and squamous cell carcinomas, and in lymphoid organs and lymphomas, further histological categorization of squamous cell carcinomas and malignant lymphomas was undertaken. The squamous cell carcinomas were categorized on the basis of characteristics considered to indicate aggressive tumour behaviour. The samples were given a score for degree of invasion and keratinization. Invasion was scored as 1 if the neoplastic cells remained *in situ* or showed only mild invasion of the superficial dermis, 2 if the neoplastic cells invaded the middermis, and 3 if the neoplastic cells were very invasive and extended to the surgical margins. The degree of keratinization was scored as 1 if most neoplastic cells were basal epithelial with little differentiation toward keratinization, 2 if the neoplastic nests had a thin layer of basal epithelium that matured centrally to prominent keratinization, and 3 if the majority of neoplastic nests and individual neoplastic cells were keratinized. The tumours were also categorized on the basis of histological patterns–invasive nests, solid sheets, cords or central comedomes with sloughed neoplastic keratinized cells. For malignant lymphomas, the tumours were further categorized into T- and B- cell immunophenotypes on the basis of labelling reactions described below.

Immunohistochemistry

Cyclin D3 expression was detected with an avidin–biotin technique and commercial mouse monoclonal antibody specific for cyclin D3. All tissues were immunolabelled with the cyclin D3 antibody, and the lymphomas, squamous cell carcinomas and basal cell tumours were also immunolabelled for the proliferation protein Ki67. Sections (5 μm) were dewaxed in xylene and in 100% ethanol. Endogenous peroxidase was blocked with hydrogen peroxide 3% in methanol for 20 min. Sections were immersed for 2 min in 95% ethanol, for 2 min in 75% ethanol, and for 5 min in tap water. Antigen retrieval was accomplished by microwaving (100 °C) in citrate-buffer for two 1-min cycles, the sections then being allowed to cool to room temperature before placing in a humidified chamber. A solution of 10% normal horse serum was applied to the sections for 20 min. After blotting, the primary antibody (NCL-Cyclin D3: Novocastra Laboratories, Newcastle-upon-Tyne, UK) diluted 1 in 20 was applied and sections were incubated overnight at 4 °C. The sections were rinsed twice (5 min each) in phosphate-buffered saline (PBS) and the secondary antibody (biotinylated horse anti-mouse; Vector Laboratories, Burlingame, CA, USA) diluted 1 in 800 was applied at room temperature for 60 min. The sections were rinsed twice in PBS (5 min each) followed by a 30-min incubation with avidin–biotin complex (ABC Elite; Vector Laboratories) diluted 1 in 50,

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