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Xenotransplantation of cryopreserved equine squamous cell carcinoma to athymic nude and SCID mice

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Abstract

Cryopreserved equine ocular squamous cell carcinoma (SCC) was inoculated subcutaneously into 15 athymic nude and 15 SCID mice. Xenotransplantation resulted in tumor growth in two athymic nude mice and 1 SCID mouse. Histological appearance and immunohistochemical characterization using cytokeratin 5/6 markers and p53 markers of the tumor grown in mice was in full accord with the original equine tumors. No evidence of metastasis was noted in any mouse. This model may serve as a relevant *in vivo* model for studying the biology of equine ocular SCC and for the testing of new therapeutic modalities.

Keywords: Equine; Horse; Squamous cell carcinoma; Cryopreservation; Nude mice; SCID mice; Xenotransplantation

Squamous cell carcinoma (SCC) is the most common neoplasm of the equine eye and ocular adnexa and the second most common tumor of the horse overall (Lavach and Severin, 1977; Junge et al., 1984). It is a locally invasive tumor with a potential to metastasize and threatens both visual outcome and long term survival of the patient. No single treatment modality has proven to be effective, and recurrence rate following surgical excision alone is as high as 62% (King et al., 1991; Mosunic et al., 2002). Much effort has been directed toward improving adjuvant therapy following surgical excision.

Murine tumor models which accurately reproduce spontaneous neoplasia play important roles in investigating tumor behavior, histological characteristics, and in evaluating efficacy of novel cancer treatments. Studies using these models provide advantages of reproducibility of the neoplasia, low cost, ease of manipulation and time effectiveness. Xenografts of animal tumors may represent metabolic characteristics of animal malignant disease and often have value in selecting tumor-specific agents when used as screening tools (Goldin et al., 1981). Xenografts established by implantation of fresh tumor tissue sections have been shown to more closely resemble the original patient specimens in their architecture and morphology compared to xenografts derived from cell lines (Shimosato et al., 1976; Hill et al., 1991; Muleya et al., 1998).

Reports of xenotransplantation of horse tumors are limited to a sarcoid derived cell line (Pardini et al., 1986) and an undifferentiated skin carcinoma (Fatemi-Nainie et al., 1984). To date, a clinically relevant *in vivo* model for equine SCC to prospectively evaluate treatment response rates to new therapeutic modalities does not exist. In the present study, we examined the ability to cryopreserve ocular SCC obtained from equine patients and the ability of SCID and nude mice to support growth of cryopreserved tumor tissue.

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Spontaneously occurring primary ocular SCC was surgically excised from three affected equine patients. Tumors in each horse were located in the third eyelid, cornea and conjunctiva. Sections of each tumor were fixed in 10% sodium phosphate buffered formalin for histologic evaluation. Remaining tumor sections were cut into 1.5 mm^3 cubes and incubated in 10 ml of TL-Hepes buffer solution supplemented with BSA and 1.5 M dimethylsulfoxide for 30 min (Bavister et al., 1983). The tissues and medium were then aspirated into a 0.5-mL freezing straw and cooled initially to −7 °C at a rate of 1 °C/min in a programmable freezer (Planer, Model: Kryo 10 Series II). At −7 °C, ice crystallization was initiated by touching the side of the straws with a cotton-tipped swab that was soaked in liquid nitrogen. After a 5 min isothermal hold, the cooling rate was decreased to 0.5 °C/min. Once the temperature reached −55 °C, the straws were transferred to liquid nitrogen for storage until implantation.

Implantation of cryopreserved equine tumor sections was performed on the dorsal lumbar subcutaneous tissues of 15 *Foxn 1^{nu}* athymic nude mice (Harlan Sprague–Dawley, IN) and 15 Fox Chase Outbred SCID mice (Charles River Laboratory, MA). Five mice of each strain were randomized to receive tumor sections from each of the three original equine patients. Transplantation was performed within 20 min following thawing of the tissues. A "take" was defined as an increase in volume of the tumor compared to the original transplantation.

Representative tumor sections from three original equine patients, cryopreserved tumor tissue and tumors from xenografted mice were stained with hematoxylin–eosin (H&E) and examined using light microscopy. Additional tumor sections were processed for immunohistochemistry using mouse anti-cytokeratin 5/6 antibody (clone D5/16B4, Zymed, South San Francisco, CA) and rabbit anti-p53 antibody (SC-6243, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The stained sections were subjectively evaluated for the presence, intensity and location of staining.

The effect of cryopreservation on cell viability in frozen-thawed tissue sections from 3 horses was evaluated with ethidium homodimer-1 and calcein AM fluorescent stains with a confocal microscope. The mean percentage (±SD) of viable cells on frozen-thawed sample was 63.3 (±8.8)%. Non-viable cells were sparsely interspersed among live cells with more non-viable cells noted at the periphery of each tissue section. The mean percentage (±SD) of viable cells in cell suspension determined by Trypan blue dye exclusion test was 61.5 (±4.1)%.

Tumor growth was observed in one recipient SCID mouse and two nude mice. Three equine tumors originating from the cornea, the third eyelid and the conjunctiva each resulted in tumor growth in one mouse. The tumors were well circumscribed solid masses located in the subcutaneous space of each mouse. The latent period between subcutaneous implantation and the first evidence of tumor growth ranged between 27 and 29 days. The tumor reached 2009.6 mm^3 in a SCID mouse at day 150. The tumors in the nude mice were 1590.9 mm^3 and 879.2 mm^3 at day 150. Tumor doubling time ranged from 5 days (at day 30) to 35 days (at day 90), with slower growth rate observed with time. Mean tumor doubling time was 14.5 days.

The cut surfaces of the xenografted tumors were homogenous grayish-white with focal, yellow, central necrotic areas. All xenografted tumors were well circumscribed with distinct fibrous capsules and were restricted to the subcutaneous space. Histopathologic evaluation of xenografted tumors confirmed well differentiated SCC with multifocal dyskeratosis, keratin pearls, and 1–3 mitotic figures per 400× field. There were no marked morphologic differences between the primary equine tumors, cryopreserved tumors and xenografted tumors. Characteristic diffuse and strong positive immunoreactivity for cytokeratin 5/6 remained the same in its intensity and location after cryopreservation and transplantation (Fig. 1). Immunoreactivity for p53 appeared as a dark brownish granular signal and was found solely in the nucleus, predominantly in the periphery of the neoplastic islands with loss of staining toward the keratinized center in excised (Fig. 2A), cryopreserved (Fig. 2B), and transplanted (Fig. 2C) ocular SCC. In all cases, tumors demonstrated only local growth without evidence of invasion into lymphatics or blood vessels. There was no evidence of metastases by visual inspection at the time of necropsy or by histopathological examination of lung, liver, kidney, spleen and regional lymph node tissue.

Primary tumor take rate reported in the present study is compatible with previous reports discussing the difficulty in establishing SCC in nude mice (Meck et al., 1981, Sharkey and Fogh, 1984). Previously reported take rates of fresh SCC xenografts were 0% for canine SCC transplanted in SCID mice (Sugimoto et al., 1994), 72% for ovine SCC transplanted in athymic nude mice (Al-Yaman and Willenborg, 1984a) and 50% and 25.9% for human SCC transplanted into SCID and nude mice, respectively (Braakhuis et al., 1984; Inohara et al., 1992). The establishment of skin SCC cell lines has been limited by the low success rate of *in vitro* culture (Al-Yaman and Willenborg, 1984b; Shimada et al., 2003). Although successful culture of normal equine keratinocytes has been reported (Dahm et al., 2002), an equine SCC cell line has not been established to date. With successful cryopreservation, tumor tissues can be thawed and implanted on an as-needed basis for development of a tumor model. Transplantable frozen samples provide a readily available tumor source without the need for repeated harvesting procedures, continuous animal passage or cell culture. In addition, xenotransplantation of cryopreserved tissue avoids the risks of biological and chemical contamination, phenotypic change, and genetic mutation that have been associated with tissue culture, further supporting these methods as useful tools in creating a murine model for equine ocular SCC (Tanaka et al., 1996; Ballo et al., 1999; Shimada et al., 2003).

The histological appearance of the xenografted SCC was in full accord with the original equine tumors with preser-