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Otarine Herpesvirus-1, not Papillomavirus, is Associated with Endemic Tumours in California Sea Lions (*Zalophus californianus*)

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Summary

The purpose of this study was to determine if Otarine Herpesvirus-1 (OtHV-1) is associated with the presence of urogenital carcinomas in California sea lions. Polymerase chain reaction (PCR) analysis with primers specific for OtHV-1 was used to compare the prevalence of OtHV-1 infection in 15 sea lions affected by urogenital carcinoma with that of age-matched and juvenile tumour-free animals, and animals with tumours of non-urogenital origin. The herpesvirus was more prevalent (100%) and more widespread in the 15 animals with urogenital carcinoma than in 25 control animals, and was most often found in the urogenital tissue (vagina and prostate) and in the draining lymph nodes. Moreover, OtHV-1 DNA was not found in any juvenile animal, or in the neoplastic tissues of animals with non-urogenital tumours. Papillomavirus-specific PCR analysis of urogenital carcinoma tissues detected papillomavirus sequences in only one carcinomatous tissue. Further studies are needed to determine if OtHV-1 contributes to oncogenesis in the California sea lion; these data show, however, that OtHV-1 is associated with urogenital carcinomas, is preferentially present in urogenital tissues, and may be sexually transmitted. Papillomaviruses, which are known to contribute to urogenital tumours in other species, did not appear to be associated with the sea lion carcinomas.

Keywords: California sea lion; gammaherpesvirus; otarine herpesvirus-1; papillomavirus; tumour; viral infection; wildlife diseases; *Zalophus californianus*

Introduction

Urogenital neoplasia is endemic in free-ranging California sea lions (*Zalophus californianus*). Over a 25-year period, neoplasia was diagnosed *post mortem* in 18.5% of sexually mature sea lions that died after stranding along the California coast; 85% of these neoplasms consisted of highly aggressive, histologically anaplastic, urogenital carcinoma (Gulland *et al.*, 1996). Moreover, subclinical benign and malignant tumours, including urogenital carcinomas, were found in 6.3% of adult female sea lions killed by harmful algal blooms (Gulland, 1999). This prevalence of neoplasia was in sharp contrast to that of an earlier survey, in which only 2.5% of stranded pinnipeds and cetaceans had tumours (Howard *et al.*, 1983). However, since that study, there have been significant reports of neoplasms in two marine organisms. First, Beluga whales (*Delphinapterus leucas*) in the St Lawrence Estuary often die of aggressive malignancies, believed to develop as a result of exposure to anthropogenic chemicals (Martineau *et al.*, 2002). Second, fibropapillomas have recently emerged as a threat to health in green and loggerhead sea turtles (*Chelonia mydas* and *Caretta caretta*), these tumours being associated with infection by a novel herpesvirus (Lackovich *et al.*, 1999).

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The high prevalence of sea lion tumours and the age class of the affected animals indicate that a combination of factors accounts for tumour development. The affected animals are sexually mature (mean age 8 years, range 5–20) but cannot be considered elderly, as sea lions can live up to 35 years (King, 1983; Gulland *et al.*, 1996). As the tumours do not occur with particular frequency in the youngest or oldest members of the population, the epidemiology is not consistent with either simple inheritance of a single defective regulator gene (as in retinoblastoma in children) or age-related accumulations of genetic mutations and deregulations (as seen in the elderly) (Cotran *et al.*, 1999). Both inheritance and senescence may contribute to sea lion tumour development; if so, however, they probably act in concert with other extrinsic factors.

Extrinsic factors that have been associated with the development of tumours in sexually mature sea lions include viruses. Herpesviral inclusions have been seen in some neoplastic cells in urogenital carcinoma and penile papilloma (Gulland *et al.*, 1996; Lipscomb *et al.*, 2000). Initial sequence data of portions of the viral terminase and polymerase genes indicate a Herpesvirus in the subfamily *Gammaherpesvirinae*. Phylogenetically, the virus, named Otarine Herpesvirus-1 (OtHV-1), clusters in the genus *Rhadinovirus*, specifically with Human Herpesvirus-8 (HHV-8), the causative agent of Kaposi's sarcoma (King *et al.*, 2002).

The occurrence of urogenital carcinomas in sexually mature sea lions is consistent with the age prevalence of virus-associated cancers in humans patients, in whom cervical cancer, aggressive forms of Kaposi's sarcoma and extra-nodal B-cell lymphoma develop in mature, but not geriatric, persons. Viral factors, encoded by human papillomaviruses and gammaherpesviruses (Human Herpesvirus-8 [HHV-8], and Epstein Barr Virus [EBV]), combine with underlying genetic predispositions or with the agents of concurrent infections to accelerate the development of neoplasia (Cotran *et al.*, 1999; Faulkner *et al.*, 2000; Goedert *et al.*, 2002; Stoler, 2003).

OtHV-1 DNA has been consistently detected in the urogenital carcinomas of adult California sea lions, but previous studies failed to determine whether the virus is (1) specifically associated with these carcinomas, (2) part of the normal sea lion flora, or (3) simply an opportunistic agent that infects the rapidly dividing neoplastic cells (Lipscomb *et al.*, 2000; King *et al.*, 2002). Furthermore, the role of papillomaviruses (causative agents of urogenital malignancies in other species) in the development of the sea lion neoplasms has not been investigated. The purpose of this study was to throw light on these questions by the use of polymerase chain reaction (PCR) analysis to detect OtHV-1 in the tissues of sea lions (1) with or without urogenital carcinomas, and (2) with other types of malignancy; and to determine whether papillomaviruses are present in the urogenital carcinomas.

Materials and Methods

Sea Lions and Samples

Adults with and without (controls) urogenital carcinomas, and juveniles. These were collected at the rehabilitation facilities of The Marine Mammal Center, Sausalito, CA and SeaWorld, San Diego, CA. In total, 40 adult and 10 juvenile sea lions were examined. Fifteen (nine female, six male) of the adults had urogenital carcinomas, and 25 (17 female, eight male) adults without tumours served as controls. No neoplasia was noted in any of the juvenile animals (eight male, two female). Animals sampled were those that died or were humanely destroyed due to poor medical prognosis. Complete gross and histopathological examinations were performed on each animal to determine cancer status and cause of death. Tissues were prepared by standard techniques for histological examination.

Fifteen tissues from each animal were collected aseptically for DNA extraction and virus detection by PCR amplification. These tissues consisted of salivary gland, tonsil, retropharyngeal lymph node, skeletal muscle, lung, liver, spleen, kidney, lumbar lymph node, urinary bladder, ovary/testis, uterus/prostate, vagina/penis, cervix/prepuce and trigeminal nerve ganglion. In the case of animals with urogenital carcinomas, the urogenital tract and draining lymph node contained grossly evident tumour tissue. The non-urogenital tissues taken from these animals were collected in areas free of grossly evident metastases. Samples were stored at −20 to −80 °C until processed.

Dentine layer patterns of the left upper canine were used to age adult animals (Oosthuizen *et al.*, 1998). In cases in which this tooth was damaged during removal, results were reported as minimum ages. Tooth ageing was provided by Kelly Robertson and Kerri Danil, Southwest Fisheries Science Center, La Jolla, CA and Pat Gearin, National Marine Mammal Laboratory, Alaska Fisheries Science Center, Seattle, WA, USA. Tooth dentine layer analysis was not performed on juvenile (i.e., non-sexually mature) animals, juvenile status being determined on the basis of physical development and standard length (nose to tail tip).

Animals with non-urogenital tumours. Thirteen such animals were identified by searching the necropsy records at the Veterinary Medical Teaching Hospital, University of California, Davis. Paraffin wax blocks containing sections of tumour tissue were sectioned into 25-μm scrolls on a Leica RM2155 Rotary Microtome (Bannockburn,

Illinois, USA), and these scrolls were used for DNA extraction. Tumour diagnosis was confirmed by examination of the archived haematoxylin- and eosin-stained slides.

DNA Extraction and PCR Analysis

DNA was extracted from fresh frozen tissues either as described by Lockridge *et al.* (1999), or with a commercially available phase lock gel DNA purification system (Eppendorf, Westbury, New York) according to the manufacturer's instructions. The tissue digestion steps of both procedures were modified to include alpha-amylase (Sigma, St Louis, MO, USA) treatment (10% by volume for 1–2 h, 37 °C) to remove the proteoglycans present in marine mammal tissues. In the case of paraffin wax-embedded tissues, DNA was extracted from two of the 25-µm thick sections (scrolls) from each tumour. Before extraction, the paraffin wax was removed from the samples by passing them through a series of xylene and alcohol rinses (Bonin *et al.*, 2003). The tissues were then processed for DNA extraction, with techniques developed for fresh frozen tissues. Additionally, to increase the quality of the DNA from the formalin-fixed tissues, extracted DNA was processed by a DNA restoration technique (Bonin *et al.*, 2003), designed to fix the small nucleic acid strand breaks that accompany formalin fixation. Use of this technique improved the outcome of subsequent PCRs. DNA samples were normalized to 50 ng/µl with a GeneQuant II spectrophotometer (Amersham Biosciences, Pharmacia Biotech, Piscataway, NJ, USA).

OtHV-1 DNA was detected in fresh frozen tissue with primers specific for a 697 bp fragment of the DNA polymerase gene (forward 5′-TTACACTTCTACGT GATGG-3′, and reverse 5′-CAATGATACTGGACGA AGA). Primers targeting a 65 bp portion of the polymerase gene (forward 5′-GCGGGAACGCAACTA TATCCT-3′; reverse 5′-CTCCCTGGTAGCCACTTG GA-3′) were designed and optimized for use on the formalin-fixed tissues. Both primer sets were developed on the basis of published sequence of the OtHV-1 DNA polymerase gene (GenBank accession no. AF236050). Reactions were performed in a 25 µl final volume with a 2.5 mM Mg concentration (Epicentre Master Mix B, Madison, WI, USA) and Qiagen HotStar Taq (Qiagen Inc., Valencia, CA, USA) for 35 cycles on a PTC 200 Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts). Cycles consisted of 40 s at 94 °C, 40 s at 60 °C and 40 s at 72 °C. A final elongation step of 10 min at 72 °C was performed after the completion of the amplification cycles. PCR products were resolved via electrophoresis through a 1.5% ethidium bromide-stained agarose gel.

PCR sensitivity was established by cloning the entire published OtHV-1 DNA polymerase sequence into a plasmid by means of the Topo TA vector kit (Invitrogen Technologies, Carlsbad, CA, USA). PCR performed on serial dilutions of these plasmid constructs determined that the reaction could detect a minimum of 256 copies of the gene. As there was neither a way in which the amount of virus could be quantified in the tissues, nor one in which OtHV-1 could be grown and quantified in culture, the plasmid experiment was used as a model for estimating approximately the sensitivity of PCR detection in fresh tissue. Sensitivity of the PCR in formalin-fixed tissue, however, could not be evaluated, since there was no way of mimicking the process of formalin-fixation and sectioning with the plasmid model. Sequencing of PCR product from 10 positive sea lions was performed with an ABI 3730 Capillary Electrophoresis Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) to ensure specificity of the amplification. This sequencing was performed by the Division of Biological Sciences Sequencing Facility, University of California, Davis.

Papillomavirus FAP59 and FAP64 primers, as described and used by Antonsson and Hansson (2002), were used to examine urogenital carcinomas from 15 animals for the presence of a 480 bp fragment of the viral L1 gene. With the HotStar Taq Master Mix Kit (Qiagen Inc., Valencia, CA, USA), and 250 ng of sample DNA, reactions were performed in a final volume of 25 µl under conditions previously described (Antonsson and Hansson, 2002). Equine sarcoid (containing bovine papillomavirus) was used as a positive control for each thermocycler run (Chambers *et al.*, 2003).

To demonstrate amplifiable DNA in the extracts, and to decrease the probability of false virus-negative results, a PCR was performed with either sea lion interleukin-2 primers (Genbank accession no. AY354470) or mammalian ferritin primers developed in our laboratory (Genbank accession no. AF246195). Since both these genes are present in all sea lion tissues, a positive result indicated that the sample DNA was of satisfactory quality for PCR.

Statistical Analysis

Mean, standard deviation, χ^2 and P values were calculated with Sigma Plot version 8 (Aspire Software International, Leesburg, Virginia) and EpiInfo 2002 version 3.2.1 (United States Centers for Disease Control, Epidemiology Program Office, Atlanta, Georgia). $P < 0.005$ was considered significant.

Results

Ages of Animals

The mean age of the urogenital carcinoma-bearing females was 9.5 years (range 7–12), and of control females

was 8.2 years (range 4–12 years). The mean age of tumour-bearing and control males was 9.6 years (range from 8 to 11) and 13.6 years (range 11–18), respectively. The true average age of the control males was likely to have been higher than reported here, as the ages of two animals could not be calculated due to cracking of the reference tooth; these two animals were reported, however, as being a minimum of 8 and 9 years old.

Viral Infection

An OtHV-1-specific amplicon was found in at least one tissue from all (nine female, six male) animals with endemic urogenital carcinomas subjected to necropsy. Virus was found most often in areas particularly affected by neoplasia, i.e., the vagina (78%), prostate (80%) and lumbar lymph nodes (60% males, 78% females) (Tables 1 and 2). Overall, virus was found in an average of 3.56 tissues per carcinoma-bearing female (range 1–6) and 4.5 tissues per carcinoma-bearing adult male (range 1–11). OtHV-1 was detected occasionally in tissue samples from the anterior parts of the carcass, e.g., retropharyngeal lymph node, skeletal muscle, lung and trigeminal ganglion.

In contrast, there was no association between the presence of papillomavirus DNA and urogenital carcinoma. Lower genital tract tissues and draining lymph nodes from all 15 tumour-bearing animals were examined. A papillomavirus amplicon 100% homologous to human papillomavirus 21 was found only in the lumbar node of a single male.

Table 1
Tissue distribution of OtHV-1 in adult female sea lions with or without urogenital carcinoma

	OtHV-1-positive samples/samples tested in sea lions	
Tissue	*without UGC (controls)*	*with UGC*
Tg ganglion	0/11	1/5 (20%)
Salivary gland	1/17 (6%)	0/9
Retropharyngeal l.n.	0/10	1/6 (16.7%)
Tonsil	0/17	0/8
Skeletal muscle	0/9	1/9 (11.1%)
Lung	0/17	1/7 (14.3%)
Liver	0/17	1/9 (11.1%)
Spleen	0/17	1/9 (11.1%)
Kidney	0/17	2/9 (22.2%)
Lumbar l.n.	0/17	7/9 (77.8%)
Urinary bladder	0/16	3/9 (33.3%)
Ovary	0/17	3/9 (33.3%)
Uterus	1/17 (6%)	5/8 (62.5%)
Cervix	0/16	5/9 (55.6%)
Vagina	0/16	7/9 (77.8%)

UGC, urogenital carcinoma; Tg, trigeminal; l.n., lymph node.
Note that the virus occurred more frequently and was more widespread in sea lions with UGC than in controls.

Table 2
Tissue distribution of OtHV-1 in adult male sea lions with or without urogenital carcinoma

	OtHV-1-positive samples/samples tested in sea lions	
Tissue	*without UGC (controls)*	*with UGC*
Tg ganglion	1/5 (20%)	1/5 (20%)
Salivary gland	0/8	1/6 (16.7%)
Retropharyngeal l.n.	0/8	1/5 (20%)
Tonsil	0/7	0/6
Skeletal muscle	0/5	1/6 (16.7%)
Lung	0/7	2 /6 (33.3%)
Liver	0/8	2/6 (33.3%)
Spleen	0/8	2/5 (40%)
Kidney	0/7	2/5 (40%)
Lumbar l.n.	0/8	3/5 (60%)
Urinary bladder	0/8	1/6 (16.7%)
Testes	0/8	0/5
Prostate	1/7 (14.3%)	4/5 (80%)
Penis	0/8	3/6 (50%)
Prepuce	1/8 (12.5%)	2/6 (33.3%)

UGC, urogenital carcinoma; Tg, trigeminal; l.n., lymph node.
Note that the virus occurred more frequently and was more widespread in sea lions with UGC than in controls.

In the tumour-free (control) adult sea lions, the OtHV-1 amplicon was detected significantly less frequently than in tumour-bearing animals ($\chi^2 = 18.77$, $P = 0.0015$), being found in only a single tissue (uterus or salivary gland) from two of 17 control females. Thus, OtHV-1 was significantly less prevalent in control females than in urogenital carcinoma-bearing females. The virus was also more widely disseminated in the carcasses of the urogenital carcinoma-bearing females, being found in a mean of 4.2 tissues (range 1–8) as compared with a mean of 0.1 (range 0–1) tissues in control females.

The difference in prevalence of OtHV-1 infection between the urogenital carcinoma-bearing and control males was also significant. OtHV-1 amplicon was detected in a single tissue (prostate, prepuce or trigeminal ganglion) from three of the eight controls (37.5%) (Table 2) ($\chi^2 = 5.83$; $P = 0.016$). Additionally, OtHV-1 DNA was more widely disseminated in the carcasses of animals with urogenital carcinoma, being detected in a mean of 5.2 tissues (range 1–11) as compared with a mean of 0.4 tissues (range 0–1) in the controls.

OtHV-1 DNA was not detected in any tissue from the juvenile sea lions, or found in any neoplastic tissue from animals with non-urogenital tumours. Table 3 summarizes the ages and diagnoses of the 13 animals with non-urogenital carcinoma. Although the two carcinomas identified bore some histological resemblance to the urogenital carcinomas, their presence in the thoracic cavity (as opposed to the urogenital system and

Table 3
Sea lions with non-endemic tumours

Animal	*Sex*	*Age class*	*Site of tumour*	*Tumour*
1	Male	Juvenile	Kidney	Fibroma
2	Female	Adult	Colon	Fibroma
3	Female	Adult	Thoracic cavity	Carcinoma
4	Male	Adult	Spleen	Haemangiosarcoma
5	Female	Adult	Thyroid	Adenoma
6	Female	Adult	Adrenal cortex	Adenoma
7	Male	Juvenile	Kidney	Fibroma
8	Female	Adult	Uterus	Leiomyoma
9	Male	Juvenile	Lymph node	T-cell lymphoma
10	Female	Adult	Cervix	Leiomyoma
11	Male	Adult	Thoracic cavity	Squamous cell carcinoma
12	Male	Adult	Thyroid	Adenoma
13	Male	Adult	Spleen	Haemangiosarcoma

abdomen) distinguished them from the endemic tumours.

Discussion

This study demonstrated, for the first time, a significant association between OtHV-1 and endemic urogenital carcinoma in the California sea lion. The virus was more prevalent and widely disseminated in the carcasses of animals with urogenital carcinomas than in those without such tumours. It was also most often detected in areas particularly affected by neoplasia. Detection of OtHV-1 DNA in sites distant from the main tumour mass may have been due to micrometastases or tumour emboli in these tissues. Limitations of the standard PCR used in this study prevented precise identification of the cells infected by OtHV-1. However, tumour cells are observed histologically in blood and lymphatic vessels distant from the areas of heavy tumour infiltration, and micrometastases are often seen in distant organs (ELB and LJL, unpublished).

The association of the virus with the urogenital carcinomas may have been due to (1) a causal relationship, (2) preferential viral infection of rapidly dividing neoplastic cells, or (3) recrudescence of latent viral urogenital infection due to disease stress. Distinguishing between these three possibilities may be difficult and has confounded studies linking Epstein Barr virus (EBV) to human mammary carcinoma. In the case of EBV, viral DNA found in mammary carcinomas led some to suspect a causal relationship. However, further studies based on quantitative PCR and analysis of viral gene expression showed that only 0.1% of the cells contained EBV DNA. Hence, the initial PCR results were due to incidental infection of the neoplastic cells by EBV undergoing lytic replication (Touitou *et al.*, 2001; Huang *et al.*, 2003).

In the present study, incidental infection of tumour cells with OtHV-1 seemed unlikely because OtHV-1 DNA was not detected in non-urogenital tumours. Significantly, OtHV-1 was not present in two aggressive carcinomas of the thoracic cavity, or in benign mesenchymal neoplasms of the urogenital tract.

The low prevalence of OtHV-1 in the control animals suggests that stress was not the reason for viral prevalence in the urogenital carcinoma-bearing animals. In theory, OtHV-1 might latently infect the urogenital tissues of all sea lions. The low viral copy number in such an infection might prevent detection by PCR and the stress of illness and rehabilitation might favour reactivation and high copy number lytic replication. Increased viral copy numbers would increase the probability of PCR detection (Croen, 1991; Flint *et al.*, 2000). All animals used in this study died or were subjected to euthanasia as a result of acute or chronic illnesses, and all were exposed to conditions that might be expected to lead to herpesviral recrudescence. However, in view of the stress experienced by both the tumour-bearing and control animals, stress-induced viral replication would seem an unlikely explanation for the findings.

The viruses most often associated with urogenital carcinomas in other species are papillomaviruses rather than gammaherpesviruses. Papillomaviruses have been shown to contribute to the development of cervical neoplasms in human beings and urinary bladder tumours in cattle (Cotran *et al.*, 1999; Borzacchiello *et al.*, 2003). In the present study, papillomaviruses were not associated with urogenital carcinomas. The papillomavirus detected in one animal was homologous to a human virus and therefore probably the result of tissue contamination during handling.

Several gammaherpesviruses have been described in other species. Some, such as HHV-8, *Herpesvirus saimiri* and Rhesus rhadinovirus, are oncogenic under certain in-vivo conditions (Damania and Jung, 2001; Fickenscher and Fleckenstein, 2001; Cotter and Robertson, 2002). Others, such as Murine Herpesvirus 68, a newly described rhadinovirus of chimpanzees, and Bovine Herpesvirus-4, have no known association with neoplasia (Lacoste *et al.*, 2000; Mistrikova *et al.*, 2000; Zimmermann *et al.*, 2001). The pathogenesis of gammaherpesvirus-induced oncogenesis is complex and the outcome of infection depends upon multiple factors. For example, EBV is highly prevalent in human populations. Normally this virus causes a self-limiting lymphoproliferative disease (Touitou *et al.*, 2001); however, when combined with the appropriate co-factors, such as concurrent malaria infection, EBV infection may result in lymphoid tumours, or nasal pharyngeal carcinoma (Touitou *et al.*, 2001).

It is not possible to determine from the data presented whether OtHV-1 has an affinity for urogenital tissue or has a limited replication environment. The prevalence of the virus in the urogenital tissue of adults and the lack of virus in juvenile animals indicate that OtHV-1 infection may be acquired through breeding activities and be limited to replication in urogenital tissues. Sexual activity is a factor in the spread of HHV-8, in which prevalence of infection is highest in those with numerous sexual partners (Gandhi and Greenblatt, 2002).

It was of interest that OtHV-1 DNA was detected in the trigeminal ganglion of three sea lions. Two of these animals had urogenital carcinoma and one did not. In the two tumour-bearing animals, infected tumour cells may have metastasized to the area. In the non-tumour-bearing animal, OtHV-1 may have become latent in ganglion cells. Neurotropic latency, which is well recognized for alphaherpesviruses, would be unusual for a gammaherpesvirus, as latent infections with such viruses are generally located in lymphoid cells. However, insufficient information is available for OtHV-1 to rule out the possibility of latent infection in neurons.

The data presented suggest but do not prove an association between OtHV-1 and urogenital carcinomas. However, the initial hints of the oncogenic potential of EBV and HHV-8 came as a result of epidemiological studies and the detection of viral DNA sequences in tumour tissues (Klein, 1998; Raab-Traub, 2002). More detailed viral genetic studies led to the discovery of oncogenic sequences in these viruses and confirmed their mechanistic role in tumour development (Damania and Jung, 2001). Similar genetic analysis of OtHV-1 remains to be carried out. A full understanding of the pathogenic significance of OtHV-1 will require (1) confirmation that OtHV-1 is present in significant numbers of neoplastic cells, (2) genetic sequencing to confirm the presence of oncogenes, and (3) the development of a serological test to determine the prevalence of OtHV-1 in sea lion populations. These initial data, however, suggest that OtHV-1 merits further study as a model of gammaherpesvirus-induced oncogenesis.

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