Oncolytic Potential of Reovirus as a Novel Therapy in Canine Cancers

(犬の腫瘍性疾患に対するレオウイルスによる

腫瘍溶解性ウイルス療法の検討)

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GENERAL INTRODUCTION

Reovirus

So far, seven taxonomic families of double-stranded RNA (dsRNA) viruses have been identified and *Reoviridae* is the largest and the most diverse in terms of host range (Schiff et al., 2007). Twelve genera are recognized in this family and each of the genera exhibit substantial genetic, structural and biological variations. The genus orthoreovirus, also known as reovirus, derives from the acronym **r**espiratory and **e**nteric **o**rphan (Sabin, 1959). Being a non-enveloped icosahedral virus with dsRNA, reovirus has ten discrete gene segments in three size classes (L, M and S), which make up a total size of approximately 23,000 base pairs. Each gene segment encodes either one or two proteins that can be transcribed into full-length mRNAs. These gene segments can undergo reassortment between virus strains.

Reovirus has a total of eight structural proteins, where the outer capsid is made up of four proteins (λ2, μ1, δ1 and δ3) and the inner capsid made up of another four (λ1, λ3, μ2 and δ2). Subviral particles (ISVPs and cores) can be generated from fully intact virion particles by proteolysis. Sialic acid (Barton et al., 2001a) and junctional adhesion molecules (JAM) (Barton et al., 2001b) can serve as cell surface receptors for recognition by cell-attachment protein δ1. Proteolytic processing of the outer capsid proteins δ3 and μ1/ μ1C is essential for infection, which can occur either extracellularly or in the endosome/ lysosomes. Since the uncoating of the parent particles is incomplete, the genomic dsRNA does not exit the particle to enter the cytoplasm and replication only occurs in the cytoplasm. Transcription and capping of viral mRNAs occur within the particles and are mediated by the particle-associated enzymes. On top of that, mature virions are inefficiently released from infected cells by lysis (Schiff et al., 2007).

Reovirus is ubiquitous in geographical distribution and has the capacity to infect nearly every known mammalian species, including humans and dogs (Rosen, 1962). Reovirus can also be isolated from a variety of fresh and seawater sources (Matsuura et al., 1984; Muscillo

et al., 2001), consistent with their pervasiveness and predominantly enteric route of transmission. Reovirus was first isolated from the human respiratory and enteric tracts, and was not associated with any serious illnesses. Serotypes of reovirus are distinguishable by means of the capacity of reovirus neutralizing antibodies to neutralize viral infectivity and inhibit hemagglutination (HA) (Rosen, 1960a; Sabin, 1959). Reovirus neutralization and HA activities are restricted to a single reovirus gene segment, S1, that encodes for the σ1 and σ1s proteins (Weiner and Fields, 1977). The prototype for reovirus type I Lang, T1L, is from an isolate from a healthy child. The prototype for reovirus type 2 Jones, T2J is from an isolate from a child with diarrhea. There are two prototypes for reovirus type 3, and both are isolated from children. They are the type 3 Dearing (T3D) isolated from diarrhea and the type 3 Abney (T3A) isolated from an upper respiratory illness (Ramos-Alvarez and Sabin, 1958; Rosen et al., 1960b; Sabin, 1959). Even though these prototypes have been studied most extensively, other strains from all three serotypes have been documented at the same time (Hrdy et al., 1979). As for dogs and cats, isolation of various serotypes and strains of reovirus has also been reported (Binn et al., 1977; Csiza, 1974; Kokubu et al., 1993; Lou and Wenner, 1963; Marshall et al., 1987; Massie and Shaw, 1966; Mochizuki and Uchizono, 1993; Scott et al., 1970).

Oncolytic viruses

It has been long believed that some pathogens have the ability to infect and destroy the cancer cells selectively. The first report of such phenomenon was in 1896 when a leukemic patient went into remission after a virus infection (Dock, 1904), and followed by another case of a boy regressing from lymphatic leukemia after a chickenpox infection (Bierman et al., 1953). Since then, research on oncolytic viruses has continued to increase as a branch of advanced cancer therapeutics. Oncolytic viruses are viruses that are capable of selectively

target and destroy tumor cells without causing damage to surrounding normal tissue. In order to do so, these viruses must be able to replicate in the targeted tumor cells to produce high titers of infectious viral progeny before cell lysis as part of direct tumor cell killing and to spread the infection to additional tumor cells.

In order to be employed successfully and safely as an anti-cancer therapeutic, it is desired that an oncolytic virus displays attributes that include a well-characterized viral genome and protein function, relatively low pathogenicity, ability to replicate specifically in tumor cells, easy genetic manipulation and does not cause serious side effects following administration (Meerani and Yao, 2010). Even though the primary mode of action for oncolytic viruses is to induce direct destruction of cancer cells, increasing evidence has suggested that these viruses further invokes the development of anti-tumor immune response (Russell et al., 2012). This immunity response can also attack the existing cancer cells and further protect the host against subsequent tumor challenges, even after the discontinuation of the therapy (Gujar et al., 2010).

Adenovirus, Coxsackie virus, herpes simplex virus, measles virus, Newcastle disease virus, parvovirus, poliovirus, reovirus, Seneca Valley virus, retrovirus, vaccinia virus and vesicular stomatitis virus are among the viruses that possess the attributes of a potential oncolytic virus. Some oncolytic viruses are naturally occurring, whilst others are genetically engineered to reduce pathogenicity, enhance tumor cell selectivity and encode therapeutic genes. These viruses have shown promising results, up to the extent of phase II and III clinical trials in human cancers, including cancer of the lung, bladder, liver, pancreas, prostate, ovaries, head and neck, glioma, melanoma and other solid tumors (Russell et al., 2012).

However, the use of oncolytic virotherapy in veterinary medicine is still far from becoming commercially available as promising laboratory results have yet to be translated

into clinical outcomes. So far, canine cancers, such as osteosarcoma, malignant melanoma, lymphoma, soft tissue sarcoma, mammary adenoma and carcinoma, have been tested with only a few oncolytic viruses, mainly adenovirus, canine distemper virus and vaccinia virus (Patil et al., 2012). Up to date, there has been no report on the feasibility of reovirus in veterinary oncology. Therefore, our laboratory is the first to report the potential of reovirus in canine cancers, with the goal of providing a better and wider range of cancer therapeutic options.

Reovirus as an oncolytic virus

Even though all three serotypes of reovirus possess oncolytic abilities (Alloussi et al., 2011), the spotlight has always been on the serotype 3 Dearing strain. This serotype of reovirus has been extensively studied in a wide range of human neoplasms such as pancreatic, bladder, gastric, breast, neurological, lung, ovarian, colon, head and neck cancers (Etoh et al., 2003; Hirasawa et al., 2002; Kawaguchi et al., 2010; Kilani et al., 2003; Norman et al., 2002; Sei et al., 2009; Twigger et al., 2012; Wilcox et al., 2001). A phase III study on the intravenous reovirus therapy in combination with carboplatin and paclitaxel in head and neck cancers is the biggest accomplishment of reovirus as a cancer therapy at this point of time (www.oncolyticsbiotech.com/clinical-trials). Even though this study was conducted on expanded groups consisting of patients with certain disease conditions, the progression free survival of patients treated with the reovirus combination therapy had a statistically significant response.

The first report on the selective cytotoxicity of reovirus in a panel of transformed cell lines dated as far back as 1977, where Hashiro et al. tested the susceptibility of various mammalian cell lines to reovirus. The second report by Duncan et al. (1978) showed that reovirus induced cell death preferentially in transformed cells but not in normal cells. Even

though both reports indicated that there was a difference in cytotoxicity of normal and tumor cells, reovirus was able to infect and produce viral progeny regardless of cell types. After the discovery of the preferential, but not definite, infection of reovirus towards transformed cells, further studies have been conducted to elucidate the mechanism behind this phenomenon.

Mechanism of reovirus oncolysis

Transformation of cell lines that were naturally resistant to reovirus with v-*erbB*, *sos* or *ras* (all activators of Ras signaling pathway) allowed the cells to be highly susceptible to reovirus infection (Strong and Lee, 1996; Strong et al., 1998). This suggested that reovirus takes advantage of the already activated Ras signaling pathway to enhance viral infectivity and replication. Further investigation led to the discovery of one of the downstream proteins of Ras to be the molecule that controls this enhancement of reoviral susceptibility. Phosphorylation of the PKR (dsRNA-activated protein kinase) inhibited the translation of viral genes and viral replication in untransformed cells while the inability of the transformed cells to phosphorylate PKR allowed reovirus to replicate freely (Coffey at al., 1998; Strong et al., 1998). Ras activation enhances the disassembly of incoming virus, viral infectivity and release (Marcato et al., 2007). These three crucial steps alone can increase the amplification of more than 100 fold of reovirus in Ras-transformed cells.

So far, correlation of reoviral susceptibility and Ras activation has been confirmed in a range of tumors such as mantle cell lymphoma (Alain et al., 2010), gastric cancer (Kawaguchi et al., 2010), breast cancer (Hata et al., 2008), pancreatic cancer (Etoh et al., 2003), glioma (Alloussi et al., 2011), ovarian and colon cancer (Hirasawa et al., 2002). It has also been reported that 90% of malignant glioma cell lines that were susceptible to reovirus infection had phosphorylation of MAPK, a downstream protein of Ras (Wilcox et al., 2001).

On the other hand, debates on the role of activated Ras in reoviral susceptibility started

when inconsistencies were found after extensive research. One of these reports argued that even though cytopathic effects (CPE) were not seen in NIH-3T3 cells in their study, the level of Ras expression was the same as the other cell lines that were susceptible to reovirus (Song et al., 2009). The authors went on to investigate the correlation of the level of phosphorylation of PKR with CPE and Ras expression, where none was established. Therefore, the authors concluded that the susceptibility of cells to reovirus does not depend on the Ras signaling pathway, but the reovirus receptor on the cell surface is the determinant factor.

Reports have also shown that reovirus can exert its oncolytic effects independent of this pathway. One of these reports highlighted that there was no correlation of the Ras and EGFR levels to reoviral susceptibility in 15 head and neck cancer cell lines (Twigger et al., 2012). Inhibition of the downstream proteins of Ras (MEK, PI3K and p38) and PKR does not alter the reovirus-induced cell death. Combination of these data indicated that the EGFR/Ras/MAPK signaling pathway is not involved in reoviral cytotoxicity in the head and neck cancer cell lines. On top of that, the mouse fibroblast cell line L929, which is highly susceptible to reovirus and often used in the production of reovirus due to its high replication efficiency, only harbors a low Ras level (Alain et al., 2007). There was also a lack of association between the susceptibility of reovirus and Ras activation in hematopoietic cancer (Thirukkumaran et al., 2003), colorectal tumor (Smakman et al., 2006; van Houdt et al., 2008) and non-small cell lung cancer (Sei et al., 2009). This highlights the complexity of the mechanism in which reovirus works in cancer cells and that our current understanding is insufficient to pinpoint a definitive biomarker of susceptibility to reovirus.

Even though there are a lot of reports on the Ras signaling pathway in the contribution of reovirus replication and spread, information on the effects of reovirus on cell signaling pathways are scarce. This information is essential to understand how reovirus utilizes

different signaling pathways to exert its effects and to find out the determinant molecule of reoviral susceptibility. One recent report has indicated that reovirus infection downregulates the activation of Ras, ERK and AKT in gastric cancer cells without the involvement of p38. These downregulated proteins, in turn, induce apoptosis in the TRAIL-resistant cells (Cho et al., 2010). Reovirus also increases the phosphorylation of the JNK-dependent transcription factor c-Jun while reducing the phosphorylation of ERK in L929 cells (Clarke et al., 2001).

Besides inducing apoptosis in cells, some viruses, such as the Varicella-zoster virus (VZV) and Adenovirus type 5, activates the Ras signaling pathway as a mean to enhance viral replication (Rahaus et al., 2006; Schumann et al., 2006). On the other hand, hepatitis C virus downregulates the Ras downstream proteins to prevent apoptosis and induce infected cells in a state of persistent infection (MacDonald et al., 2003) while Herpes Simplex virus 1 does so to increase viral resistance and to prevent further infection (Georgopoulou et al., 2003). These phenomena show that all viruses employ different strategies to optimize their survival in the host cells.

Natural infection of reovirus

Seroepidemiological surveys of reovirus in healthy humans revealed that the incidence of seropositivity rises from approximately 35% in early childhood, to approximately 60% in teenage years and more than 85% in late adulthood (Lerner et al., 1962; Selb et al., 1994; Tai et al., 2005). However, unlike in humans, seroepidemiological data of reovirus in healthy dogs are limited. Reports have indicated that 14-63% of sampled dog populations have an elevated reovirus neutralizing antibody titer (Decaro et al., 2005; Fukumi et al., 1969; Murakami et al., 1975; Osterhaus et al., 1977). It has been reported that the dramatic increment of reovirus neutralizing antibody hampers the efficiency of intravenous reovirus therapy in human cancer patients (White et al., 2008). Similarly, in canine cancer patients, a

high reovirus neutralizing antibody titer due to natural infection might also negatively affect reovirus therapy. Therefore, the data on the seroepidemiology of reovirus in the dog population is important to allow a sound prediction of the effects of this therapy in canine cancers.

Clinical trials involving oncolytic viruses and reovirus

The first clinical trial using virus as treatment for cancer was recorded in 1949, where sera and tissue containing Hepatitis B virus were injected into 22 patients with Hodgkin's disease. The outcome was fair, with 7 patients who improved in clinical aspects of the disease and 4 patients had a reduction in tumor size. On the contrary, more than half of the patients developed hepatitis due to virus infection. Another three clinical trials involving the West Nile virus, adenovirus and mumps virus, were conducted within the next few years with variable results (Kelly and Russell, 2007). It has been a long way since then when the world's first oncolytic virus therapy using the adenovirus H101 was approved by China in 2005 (Garber, 2006).

On the other hand, the first clinical trial involving reovirus was completed in 2002, 53 years after the first clinical trial using an oncolytic virus. Even though a late bloomer, Reolysin, the reovirus serotype 3 Dearing strain which is developed as an oncotherapy by Oncolytics Biotech Inc., has reached as far as a total of 32 completed and ongoing clinical trials up-to-date, involving melanoma, sarcoma, multiple myeloma, solid tumor, colorectal, prostate, pancreatic, lung, ovarian, brain, breast, head and neck cancer. These studies included 11 phase I, four phase I/II, 14 phase II, two translational and one phase III clinical trial (www.oncolyticsbiotech.com/clinical-trials).

Objectives of this doctoral thesis

Seeing that reovirus has been tested fervently in human cancers, we chose reovirus as the oncolytic virus to focus in our studies. We strongly believe in the ability of reovirus to be one of the next generation cancer therapeutics of the highest potential. Being a naturally occurring virus that requires no genetic engineering, the natural susceptibility of reovirus towards transformed cell can be harvested without the fear of potentially creating a virus that can do more harm than good. As the life cycle of reovirus takes place in the cytoplasm of host cells and does not include a stage of viral DNA synthesis (Schiff et al., 2007), there won't be any possibility of incorporation of viral genome fragments into the host cell DNA and cause disastrous mutations. Even though reovirus can replicate and produce high viral progeny titers in susceptible cells, it is quite amazing that this virus only induces minimal toxicities, which are well tolerated by many human patients. This has allowed reovirus to outshine the other oncolytic viruses in many ways.

Approximately 30-40% of all human cancers have been reported to have Ras activating mutations, with the highest incidences found in pancreatic cancer (90%), colon cancer (50%), thyroid tumors (50%), lung cancer (30%) and myeloid leukemia (30%) (Bos, 1989). However, unlike human cancer, it has been known that mutation in *ras* itself is uncommon in canine cancers (Escobar et al., 2004; Griffey et al., 1998; Mayr et al., 2002; Watzinger et al., 2001). On the other hand, naturally occurring cancers of dogs and humans have been reported to be alike not just in terms of histopathology, progression, physiologic and systemic effects of the tumor, but also commonly involve the same genes and pathways which exhibit similar response to cancer therapies (Gordon and Khanna, 2010; Paoloni and Khanna, 2007; Paoloni and Khanna, 2008). Therefore, we hypothesize that similar cytotoxic effects of reovirus as seen in many human cancers, could also be displayed in canine cancers.

Armed with confidence based on essential information from previous studies, goal-

oriented objectives were carried out step by step throughout my PhD studies to meticulously assess the feasibility of reovirus (serotype 3 Dearing strain) in canine cancers. The results of all these objectives where discussed in further details in five chapters. In Chapter 1, the cytotoxic effects of reovirus were examined in canine mast cell tumor (MCT) cell lines before assessment *in vivo*, *ex vivo* and in bone marrow-derived cultured normal mast cells. The cell death pathway and the involvement of the Ras activation status in the reovirus susceptible cells were also investigated. Chapter 2 reports the screening of the effects of reovirus in a panel of ten canine lymphoma cell lines and their correlation with the Ras activation status. The highlights in Chapter 3 comprise of the effects of reovirus on the Ras signaling pathway, the PI3K/Akt pathway and c-Kit, as well as the involvement of various Ras downstream apoptotic proteins. The analysis and results of the seroepidemiology of reovirus in healthy dogs were discussed in Chapter 4. The final chapter, Chapter 5, involves the experimental infection of reovirus in healthy dogs and the administration of reovirus in five canine cancer patients. Information on the dosage of reovirus, route and frequency of administration were included, together with the observed adverse events and analysis of the kinetics of viral shedding, neutralizing anti-reoviral antibodies and assessment of disease progression.

All in all, this thesis provides the basic yet essential data on the feasibility of reovirus as therapy in canine cancer. Much is left to be done in examining the susceptibility of reovirus in other types of canine cancer, elucidating the involvement of other mechanism of reovirus oncolysis, as well as conducting clinical trials to obtain the maximal tolerable dose and further assess the toxicities of reovirus in canine cancer patients. The phrase “from bench to bedside” has definitely been critically applied during my Phd studies. Hopefully with the completion of my PhD, reovirus will also have a chance to debut as a novel therapy in canine cancers.

CHAPTER 1

Effects of reovirus in canine mast cell tumor (MCT)

in vitro, *in vivo* and *ex vivo*

SUMMARY

This chapter reports the oncolytic effects of reovirus in canine mast cell tumor (MCT), the most common cutaneous tumor in dogs. MCT cell lines were highly susceptible to reovirus as indicated by marked cell death, high production of progeny virus and virus replication. Reovirus induced apoptosis in the canine MCT cell lines with no correlation to their Ras activation status. *In vivo* studies were conducted using unilateral and bilateral subcutaneous MCT xenograft models with a single intratumoral reovirus treatment and apparent reduction of tumor mass was exhibited. Furthermore, cell death was induced by reovirus in primary canine MCT samples *in vitro*. However, canine and murine bone marrow-derived mast cells (BMCMC) were also susceptible to reovirus. The combination of these results supports the potential value of reovirus as a therapy in canine MCT but warrants further investigation on the determinants of reoviral susceptibility.

INTRODUCTION

Cancer is the leading cause of human death in developed countries and this number is projected to rise each year (Jemal et al., 2011). Even though precise estimates of global cancer morbidity and mortality rates in pets are unavailable due to the lack of census, cancer has also been ascertained to be a major cause of death in pets. Furthermore, there has been an upward trend in the number of pets dying of cancer, partly due to pets living to an increasingly older age and improved veterinary care (Butler et al., 2013). Therefore, there is a dire need for more advanced and effective animal cancer therapy.

Mast cell cancer is rare in humans (Metcalfe, 2005) but mast cell tumor (MCT) is the most common cutaneous tumor in dogs, comprising approximately 16% to 21% of all canine cutaneous tumors (London and Thamm, 2013). Complete surgical excision is potentially curative in well-differentiated and intermediate grade canine MCT while radiation or medical therapy is often necessary as adjunctive therapy for incompletely resected tumors. However, undifferentiated canine MCT is an aggressive tumor that frequently metastasizes to local lymph nodes, spleen, liver, and possibly to the bone marrow and peripheral blood. Most dogs with the aggressive form of the tumor die within one year of diagnosis. Therefore, new therapeutic approaches to canine MCT are needed.

Despite the fact that mutation in *ras* itself is uncommon in canine cancers (Murua Escobar et al., 2004; Watzinger et al., 2001), we hypothesize that canine cancers are susceptible to reovirus as naturally occurring cancers of dogs and humans have many similarities (Gordon and Khanna, 2010). In this chapter, we examined the oncolytic effects of reovirus in canine MCT *in vitro*, *in vivo* and *ex vivo*. We also examined the relationship between reoviral susceptibility and the Ras activation status in the MCT cell lines.

MATERIALS AND METHODS

Cell cultures and reovirus

Four established canine MCT cell lines, VIMC, CoMS, CM-MC and HRMC, were used in this study. VIMC (Takahashi et al., 2001) and CoMS (Ishiguro et al., 2001) were derived from visceral MCT while CM-MC (Takahashi et al., 2001) and HRMC (Ohmori et al., 2008) were MCT of cutaneous origin. RBL-2H3, a rat basophilic leukemia cell line, and P815, a mouse lymphoblast-like mastocytoma cell line, were selected as comparison models of mast cell across species while the human Burkitt's lymphoma cell lines, Daudi and Raji, were used as negative and positive controls respectively (Alain et al., 2002). Mouse L929 fibroblastic cell line was used in the titration of progeny virus. RBL-2H3, P815, Raji and L929 were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). All cells were maintained in R10 complete medium (RPMI1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol) kept at 37˚C in a humidified 5% CO_2 incubator.

The Dearing strain of reovirus serotype 3 (Reolysin®; clinical grade reovirus; GMP) was obtained from Oncolytics Biotech Inc. (Calgary, Canada) through a collaborative effort. Ultraviolet (UV)-inactivated virus was prepared by exposing live virus to UV light for 90 minutes.

Reovirus infection of cell lines

VIMC, CoMS, CM-MC, HRMC, Daudi and Raji were seeded at 2.5 x 10^4 cells while RBL-2H3 and P815 were seeded at 1.25 x 10^4 cells before being mock-infected or infected with reovirus at multiplicity of infection (MOI) of 70 plaque-forming units (PFUs) per cell. All cells were seeded in triplicates in 48-well plates. Cells were stained with 0.25% trypan

blue and viability counted via hemocytometer at 72 hours post-infection (hpi). Supernatant from each sample was collected and kept at -80 ˚C, pending titration of progeny virus using 50% tissue culture infectious dose ($TCID_{50}$) assay on L929 cells, as previously described (Reed and Muench, 1938) with modifications.

The same cell number of canine MCT cells was also used to assess the cell viability curve at 0, 24, 48 and 72 hpi at MOI 70 of reovirus. To assess the sensitivity of cells towards various titer of reovirus, the same cell number of canine MCT cells was incubated with UV-inactivated, 2.8, 14 and 70 MOI of reovirus for 72 hours before cell viability and titer of progeny virus were assessed.

Reovirus infectivity and Poly (ADP-Ribose) Polymerase cleavage (PARP)

Canine MCT cells were seeded at 5.0 x 10^5 and mock-infected or infected with reovirus at MOI 70 for 6 and 24 hours. The same number of control cells (Daudi and Raji) was mock-infected or infected with reovirus at MOI 70 and cells were harvested at 6, 24 and 48 hpi. Whole cell lysates for reovirus infectivity were treated with RIPA lysis buffer (50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate) while lysates for PARP cleavage were lysed with NP40 lysis buffer (1% NP40, 10 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA), both supplemented with complete, Mini EDTA-free protease inhibitor mixture (Roche Diagnostics K.K., Tokyo, Japan). Proteins were subjected to SDS-PAGE and Western blotting.

Measurement of cell death by propidium iodide (PI) staining

Canine MCT cells that were seeded at 1.0 x 10^5 were infected with reovirus at MOI 70 and cells were harvested at 48 and 72 hpi. Cells were washed with cold PBS and fixed with cold ethanol before storage at -20 ˚C, pending analysis. Cells were incubated with 100 µl of

100 μg/ml Ribonuclease A (Nalacai Tesque, Kyoto, Japan) for 30 minutes before 4 μl of 1 mg/ml PI (Sigma-Aldrich Japan K.K., Tokyo, Japan) was added 5 minutes before acquisition. Flow cytometry was performed using CyFlow® Space (Partec GmbH, Münster, Germany) and results were analyzed using FlowJo software (Tree Star, Inc., San Carlos, CA, USA). The percentage of subG1 cells was considered as apoptotic cells.

Inhibition of reovirus cell killing by Z-VAD-FMK

To provide further evidence that reovirus-induced cell death was due to apoptosis, 2.5 x 10^4 cells were seeded in triplicate and pre-treated with control DMSO, 10 μM or 100 μM of Z-VAD-FMK (caspase inhibitor I; Calbiochem, Billerica, MA, USA) for 30 minutes at 37˚C before being infected with reovirus at MOI 70. Viable and non-viable cells were assessed using 0.25% trypan blue at 48 hpi.

GST pull-down assay for Ras status

Ras activation status of each cell line was evaluated after GST pull-down (Taylor et al., 2001). To construct a vector that expresses GST fused with Ras-binding domain (RBD) of Raf-1, a part of Raf-1 gene encoding amino acids 1-149 of Raf-1 was amplified using plasmid pCMV5 Raf-1 as a template and primers YTM647 (5'-CGTGGA TCCGAGCACATACAGGGAGCT-3', underline indicates the BamHI site) and YTM648 (5'-CGGGAATTCAGCTTCAGGAACGTCTT-3', underline indicates the EcoRI site) as previously described (Umeki et al., 2011). The amplified PCR products were subcloned into the BamHI and EcoRI sites of the pGEX 4T-3 vector (pGEX-RBD#2). JM109 was transformed with pGEX-RBD#2 and GST-RBD was extracted with lysis buffer. Cytoplasmic extract from cells (300μg) was mixed with glutathione-Sepharose 4B beads (GE Healthcare, Tokyo, Japan) conjugated with GST-RBD protein for 1 hour before washing with lysis buffer.

Precipitated Ras-GTP and whole cell lysates were subjected to SDS-PAGE, followed by Western blotting.

Western blotting

Following electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and probed with specific primary antibodies as follows: rabbit anti-reovirus (produced by our lab), rabbit anti-PARP (NeoMarkers, Fremont, CA, USA) or mouse anti-pan-Ras (Calbiochem). Incubation with primary antibodies was followed by secondary labeling using goat anti-rabbit or goat anti-mouse IgG HRP (Zymed Laboratories, San Francisco, CA, USA). The membranes were visualized by immersion in Western Lightning Chemiluminescence reagent (PerkinElmer, Shelton, CT, USA). Immunoreactive bands were visualized using the Luminescent Image Analyzer LAS 3000 mini (FUJIFILM, Tokyo, Japan) and analyzed using Science Lab 2005 (FUJIFILM). Membranes were stripped between antibody staining procedures with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl (pH 6.7)) for 30 minutes at 60 ˚C. Goat anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit anti-goat IgG HRP (Bethyl Laboratories, Inc., Montgomery, TX, USA) were used as loading controls.

Subcutaneous tumor xenograft models in NOD/SCID mice

Eight to nine-week-old NOD/ShiJic-*scid* (NOD/SCID) mice were obtained from Kyudo Co. Ltd. (Saga, Japan) and studies were conducted in a specific pathogen-free area. All animal procedures were conducted in accordance to the Yamaguchi University Animal Care and Use guidelines and were approved by the Institutional Animal Care and Use Committee of Yamaguchi University (Permit Number: 188). All efforts were made to ensure minimal pain and suffering of the animals.

VIMC or CoMS cells (1.0 x 10^7 in 50 μl PBS) were implanted subcutaneously into one or both flanks of the mice under general anesthesia. When the desirable tumor size was achieved on either side, 7.0 x 10^7 PFUs of live reovirus (experimental group) or UV-inactivated reovirus (control group) in 20 μl PBS were injected intratumorally. Two-dimensional tumor measurements were performed with a caliper every other day until euthanasia due to excessive tumor burden. Tumor measurements were analyzed and shown as tumor mass (mm^3). Tumors and remaining masses were fixed in 10% neutral buffered formalin and embedded in paraffin before staining with hematoxylin and eosin (H&E) for histopathological analysis.

For immunohistochemical (IHC) staining, deparafinized samples were treated with Target Retrieval Solution (Dako, Glostrup, Denmark) before treatment with 3% hydrogen peroxidase and Protein Block (Dako). Sections were then incubated with rabbit anti-reovirus polyclonal antibody (1:500 dilution; produced by our lab), followed by Histofine Simple Stain MAX-PO (R) (Nichirei Biosciences, Inc., Tokyo, Japan). Slides were subjected to 3,3-diaminobenzidine tetrachloride (Roche Diagnostics K.K., Tokyo, Japan) staining before counterstaining with Meyer's hematoxylin.

Reovirus infection of primary canine MCT samples

Primary canine MCT tumor cells were obtained by fine needle aspiration (FNA) from canine patients with confirmed diagnosis of MCT at the Yamaguchi University Animal Medical Center. Immediately after collection, 2.5 x 10^4 cells were seeded in triplicate before being mock-infected or infected with reovirus at MOI 70. Viability of cells was assessed at 72 hpi with 0.25% trypan blue and progeny virus was measured using $TCID_{50}$ assay.

Generation of canine and murine bone marrow-derived cultured mast cells (BMCMC)

Canine BMCMC was isolated and purified from bone marrow cells of two healthy beagles as previously reported (Kawarai et al., 2009; Lin et al., 2006). Briefly, bone marrow cells were separated using Lymphoprep™ kit (Axis-Shield PoC AS, Oslo, Norway) before $CD34^+$ cells were enriched using MidiMACS (Miltenyi, Auburn, CA, USA) separator. $CD34^+$ cells were cultured in Stemline II (Sigma-Aldrich) supplemented with 100 ng/ml recombinant canine stem cell factor (rcSCF; R&D Systems, Minneapolis, MN, USA) and incubated at 37˚C in a humidified 5% CO_2 incubator. Matured $CD117^+$ cells were enriched with MidiMACS separator again 4 weeks after culture.

Murine BMCMC was generated from bone marrow cells of C57BL/6N mice (Kyudo Co. Ltd.) and cultured in D10 (DMEM with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol) supplemented with murine IL-3 (PeproTech Inc., Rocky Hill, NJ, USA) (Kalesnikoff and Galli, 2011). Cultures were grown at 37˚C in a humidified 5% CO_2 incubator for 5 weeks before $CD117^+$ cells were enriched with MidiMACS separator.

Purity of canine and murine BMCMC were confirmed with toluidine blue staining after attaching BMCMC on glass slides using cytospin and fixing in Carnoy's solution for 30 minutes. Both canine and murine BMCMC were seeded in triplicate before being mock-infected or infected with reovirus at MOI 70 and cell viability was assessed at 72 hpi with 0.25% trypan blue. Progeny virus produced was titered using $TCID_{50}$ assay.

RESULTS

MCT cell lines are susceptible to reovirus-induced cytotoxicity in vitro

The susceptibility of MCT cell lines to reovirus infection and oncolysis was first examined with MOI 70 of reovirus and cell viability was assessed at 72 hpi. All MCT cell lines, especially the canine MCT cell lines, were susceptible to reovirus (Fig. I-1A; $p<0.05$). At 72 hpi, 100% cell death was induced in VIMC, CoMS and CM-MC and more than 80% was induced in HRMC. Reovirus-infected P815 showed a higher percentage of cell death in comparison to RBL-2H3. Cell viability was also reduced ($p<0.05$) in Raji, but not in Daudi.

Following reovirus infection, P815, VIMC, CoMS, CM-MC, HRMC and Raji produced a significant amount of progeny virus at 72 hpi, while Daudi did not (Fig. I-1B). This indicates that reovirus is able to replicate in susceptible cell lines and infectious progeny virus is released for subsequent infection cycles. Even though progeny virus was produced in RBL-2H3, the amount was not significantly higher than the input titer. Examination of the morphology of the canine MCT cell lines revealed distinct virus-induced cytopathic effects (CPE) at 72 hpi in contrast to the mock-infected cells (Fig. I-1C).

Reovirus is highly infective in canine MCT cell lines

The kinetics of reovirus infection in canine MCT cell lines at 0, 24, 48 and 72 hpi at MOI 70 was assessed. By 48 hpi, 100% cell death was seen in VIMC and CoMS while more than 95% and 45% cell death was detected in CM-MC and HRMC (Fig. I-2A). This indicates that canine MCT cell lines were highly susceptible to reovirus, with VIMC and CoMS being the most susceptible, followed by CM-MC and HRMC.

When the canine MCT cell lines were inoculated with UV-inactivated, 2.8, 14 and 70 MOI of reovirus, 100% cell death in VIMC and CoMS was induced at MOI as low as 2.8

PFUs/ cell. At the same MOI, more than 95% and 50% cell death was induced in CM-MC and HRMC respectively (Fig. I-2B). Measurement of the titer of progeny virus produced in the cell lines revealed that the titer was comparable among the various groups of MOI, suggesting that the amount of virus used in the initial infection does not play a critical role in inducing cytolysis in reovirus susceptible MCT cell lines (Fig. I-2C). No progeny virus was detected in the UV-treated group.

To confirm the infectivity of canine MCT cell lines to reovirus, detection of the reovirus μ and σ protein was carried out via Western blotting. The synthesis of both the μ and σ protein was detected as early as 6 hpi in all the canine MCT cell lines as compared to P815, RBL-2H3 and Raji (Fig. I-2D), suggesting that reovirus replicates more efficiently in canine MCT cell lines. Synthesis of reovirus protein was not detected in Daudi even up to 48 hpi, confirming that Daudi is resistant to reovirus.

Reovirus induces apoptosis in MCT cell lines

Confirmation of cell death in canine MCT cell lines was subsequently assessed with PI staining. An increment in the proportion of the subG1 cells indicated that the number of apoptotic cells increased from 48 to 72 hpi. At 72 hpi, more than 95% cell death was seen in VIMC and CoMS, 75% in CM-MC and 48% in HRMC (Fig. I-3A). The consistency of these results indicates that although trypan blue is usually used as a fast and easy screening of cell viability, it is a reliable technique to directly display the proportion of viable and non-viable cells.

Western blot analysis of PARP cleavage in the canine MCT cell lines confirmed the appearance of the signature cleavage product as early as 6 hpi in VIMC and CoMS. Cleaved PARP was visualized in all the canine MCT cell lines at 24 hpi as compared to the mock-infected samples (Fig. I-3B). The cleavage of cellular substrate PARP in reovirus-infected

cells demonstrated the morphological hallmark of apoptosis.

Canine MCT cell lines were also pre-treated with Z-VAD-FMK before infection with reovirus and cell viability was determined at 48 hpi. Reovirus-induced cytotoxicity was reversed by Z-VAD-FMK in all the cell lines where cell death was slightly inhibited at 10 μM and almost completely inhibited at 100 μM (Fig. I-3C). The combination of these results allows us to conclude that reovirus induces canine MCT cell death predominantly through an apoptotic pathway.

Ras is not activated in MCT cell lines

Ras activation has been reported to contribute to proteolytic reovirus disassembly as well as increased progeny virus infectivity and release (Marcato et al., 2007). Since reovirus has not been tested in any canine cancer, it remains unclear if the Ras signaling pathway plays a role in reoviral susceptibility in these cells. Using Raji as the standard of Ras activation, our results showed that P815 and HRMC have elevated Ras activity, while RBL-2H3, VIMC, CoMS and CM-MC did not (Fig. I-4). Even though all of the MCT cell lines were susceptible to reovirus, the level of activated Ras expression varied. The notion that reoviral susceptibility is dependent on the Ras activation status is inconsistent, especially when compared to the three canine MCT cell lines that are the most susceptible to the effects of reovirus. This finding indicates that reovirus exerts its potent oncolytic effects in MCT cell lines independent of the Ras signaling pathway.

Reovirus induces regression of MCT mass in vivo

To assess the therapeutic potential of reovirus *in vivo*, VIMC and CoMS unilateral subcutaneous xenograft models were established in NOD/SCID mice and treated with a single intratumoral of reovirus injection. All mice treated with reovirus experienced

significant regression of tumor mass by day 4 post-treatment for VIMC and by day 6 post-treatment for CoMS (Fig. I-5A and I-5B; $p<0.05$). However, black tail syndrome, a possible side effect in reovirus-infected NOD/SCID mice (Loken et al., 2004), was observed in one out of five in VIMC and three out of five in CoMS-transplanted mice that were treated with reovirus.

Anticipating that reovirus-susceptible tumor would allow generation of adequate progeny virus for hematogenous dissemination, bilateral cutaneous VIMC xenograft models were created and treated with a single unilateral intratumoral reovirus injection on either mass. As expected, significant tumor regression was not only limited to the intratumoral-treated tumor, but was also observed in the non-treated contralateral tumor (Fig. I-6A; $p<0.05$). This suggests that a substantial amount of reovirus was produced and released from the treated tumor into the blood circulation. No side effects were observed in this group of mice.

In contrast to the UV-inactivated reovirus treated tumors (Fig. I-6B), H&E-stained histopathological samples from the bilateral VIMC xenograft models showed extensive necrotic lesions within reovirus-treated tumors (Fig. I-6C), confirming the presence of anti-cancer activity. Similar extensive necrotic lesions were also seen in the histopathological samples of the unilateral xenograft models (data not shown). IHC staining demonstrated the presence of reovirus proteins only in the reovirus-treated and the contralateral tumors (Fig. I-6C).

Primary canine MCT, normal canine and murine BMCMC are susceptible to reovirus

The next step of our study was to determine reovirus oncolysis in primary canine MCT samples. Reovirus induced approximately 80% cell death in both the primary MCT samples tested and an increased amount of progeny virus was produced (Fig. I-7). This strongly

supports the feasibility of using reovirus as a potential therapeutic option in cases of canine MCT.

To assess the effects of reovirus in normal mast cells, canine and murine BMCMC were generated and infected with reovirus. Cell viability was markedly reduced in the canine BMCMC (~90% cell death) (Fig. I-8A) as compared to the murine BMCMC (~40% cell death) (Fig. I-8C). Consequently, reovirus-infected canine BMCMC produced a higher titer of progeny virus as compared to murine BMCMC (Figs. I-8B and I-8D), suggesting that even normal mast cells are able to support reovirus replication. These results show that reovirus does not have an infectivity preference towards normal healthy or abnormal tumorous mast cells.

DISCUSSION

In this chapter, the susceptibility of canine MCT *in vitro*, *in vivo* and *ex vivo* towards the oncolytic effects of reovirus was examined. We demonstrated that MCT cell lines are susceptible to reovirus, especially in canine MCT. Moreover, these *in vitro* findings were confirmed *in vivo*, whereby LuMC and CoMS subcutaneous tumors underwent marked regression after a single intratumoral reovirus injection. The susceptibility of primary canine MCT cells towards reovirus further supported our hypothesis. We also discovered that normal mast cells derived from bone marrow of dogs and mice are susceptible to reovirus.

Up till now, the key mechanism that determines reoviral susceptibility has not yet been ascertained. Previous report suggests that the Ras signaling pathway plays a vital role in enhancing reovirus disassembly, infectivity and release of progeny virus (Marcato et al., 2007). Our data, however, strongly suggests that the susceptibility of MCT cell lines does not correlate with the level of activated Ras expression. This was evident in the three canine MCT cell lines that were the most susceptible to reovirus but did not have any activated Ras expression. On the other hand, reovirus did not have such a dramatic effect on HRMC and P815 even though they expressed a high level of activated Ras. Even though a concrete conclusion could not be made due to the limited number of cell lines examined, it is possible that there is a negative correlation between Ras activation and reoviral susceptibility. This negative correlation was also suggested by Twigger et al. (2012), where the oncolytic effects of reovirus was shown to be independent of the epidermal growth factor receptor (EGFR)/ Ras signaling pathway in head and neck cancer cell lines. Furthermore, the lack of association between reoviral susceptibility and the Ras signaling pathway has also been reported in human hematopoietic cancers (Thirukkumaran et al., 2003), colon cancer (van Houdt et al., 2008) and non-small cell lung cancer (Sei et al., 2009). Moreover, it is known that the *ras* mutation in canine tumors is rare (Murua Escobar et al., 2004; Watzinger et al.,

2001). Thus, it was not surprising that three out of four canine MCT cell lines that were susceptible to reovirus did not have an activated Ras signaling pathway.

Research on reovirus over the past decade have shown that it is very unlikely that the susceptibility of tumor cells towards reovirus lies solely on the simple generic activation of Ras (Barton et al., 2001; Chappell et al., 1997; Cho et al., 2010; Errington et al., 2008; Kim et al., 2010; Norman et al., 2004; Pan et al., 2013). Reovirus triggers apoptosis in a caspase-dependent manner (Clarke et al., 2004), which is also reported in this study. Other reports have indicated that reovirus induces apoptosis via the activation of cellular stress kinase, c-Jun N-terminal kinase and nuclear factor kappa B (Clarke et al., 2004; Connolly et al., 2000). Hence, there is a possibility that one of these pathways might be the key determinant of reoviral susceptibility.

So far, KIT, a receptor tyrosine kinase, has been the best-described molecular abnormality in canine MCT (London and Thamm, 2013). Normally expressed on cells such as hematopoietic stem cells, melanocytes and mast cells, KIT regulates apoptosis, cell differentiation, proliferation, chemotaxis and cell adhesion after being activated by stem cell factor (Roskoski, 2005). KIT has also been reported to play an equally important role in various human cancers (Heinrich et al., 2002). All of the canine MCT cell lines used in this study have aberrant autophosphorylation of the c-Kit receptor where VIMC, CoMS and CM-MC have KIT mutations and HRMC has a SCF/c-Kit receptor autocrine mechanism (Takeuchi et al., 2012). Since all the canine MCT cell lines are susceptible to reovirus, we speculated that the aberrant autophosphorylation of the c-Kit receptor might somehow be involved. However, from this study, we discovered that normal canine and murine mast cells are susceptible to reovirus. Therefore, it is unlikely that the c-Kit receptor contributes to reovirus-induced cell death.

On top of that, the finding that normal mast cells are susceptible to reovirus contradicts

the belief that oncolytic viruses only infect and kill cancerous cells while leaving normal cells unharmed in a selective fashion. In a recent report (Adair et al., 2012), normal mature cells, mainly the peripheral blood mononuclear cells (PBMCs), granulocytes and platelets, were found to be involved in reovirus delivery to the target cancer cells. Normal cells, such as dendritic cells, have also been cited to play an important role in transportation of reovirus to the target tumor cells in order to evade the anti-viral immune response (Ilett et al., 2011). However, this phenomenon does not involve reovirus replication in the dendritic cells (Errington et al., 2008). In our study, reovirus replication occurred in normal mast cells but the mechanisms remain unknown. Nonetheless, there is no data to suggest that reovirus poses a significant danger to dogs or other mammals. The safety implications and virus shedding after intentional reovirus infection in dogs are currently under investigation.

The host immune system is an important factor that will dictate the outcome of virotherapy. Unfortunately, unlike in the human population, limited data is available regarding reovirus infection and antibody production in dogs (Decaro et al., 2005; Fukumi et al., 1969; Murakami and Kato, 1975). Therefore, prediction of the effects of pre-existing neutralizing antibodies in reovirus therapy in dogs is difficult. Immunocompromised mouse models were also used in this study and therapeutic response might be different in immunocompetent hosts. However, the efficacy of oncolytic virotherapy in immunocompetent models has been well documented (Prestwich et al., 2009). Instead of limiting therapy, reovirus have been reported to stimulate the innate and adaptive immune response against tumor cells, acting as immunotherapy to amplify the anti-tumor effects (Errington et al., 2008a; Errington et al, 2008b; Prestwich et al., 2009). Nevertheless, the correlation between these laboratory results and the therapeutic efficacy of reovirus in a clinical setting has yet to be established. Therefore, clinical trials in canine MCT patients are necessary to establish the efficacy of reovirus in canine cancers and to evaluate the extent of

immune system involvement.

The feasibility of reovirus therapy in canine MCT is evident with the *in vitro*, *in vivo* and *ex vivo* data obtained in this study. However, there remains a need to clarify the mechanism behind the cytotoxic effects of reovirus in canine MCT as blind usage of reovirus could have disastrous consequences. In summary, as in human cancers, reovirus has great potential as a next generation therapeutic option in veterinary oncology.

FIGURES

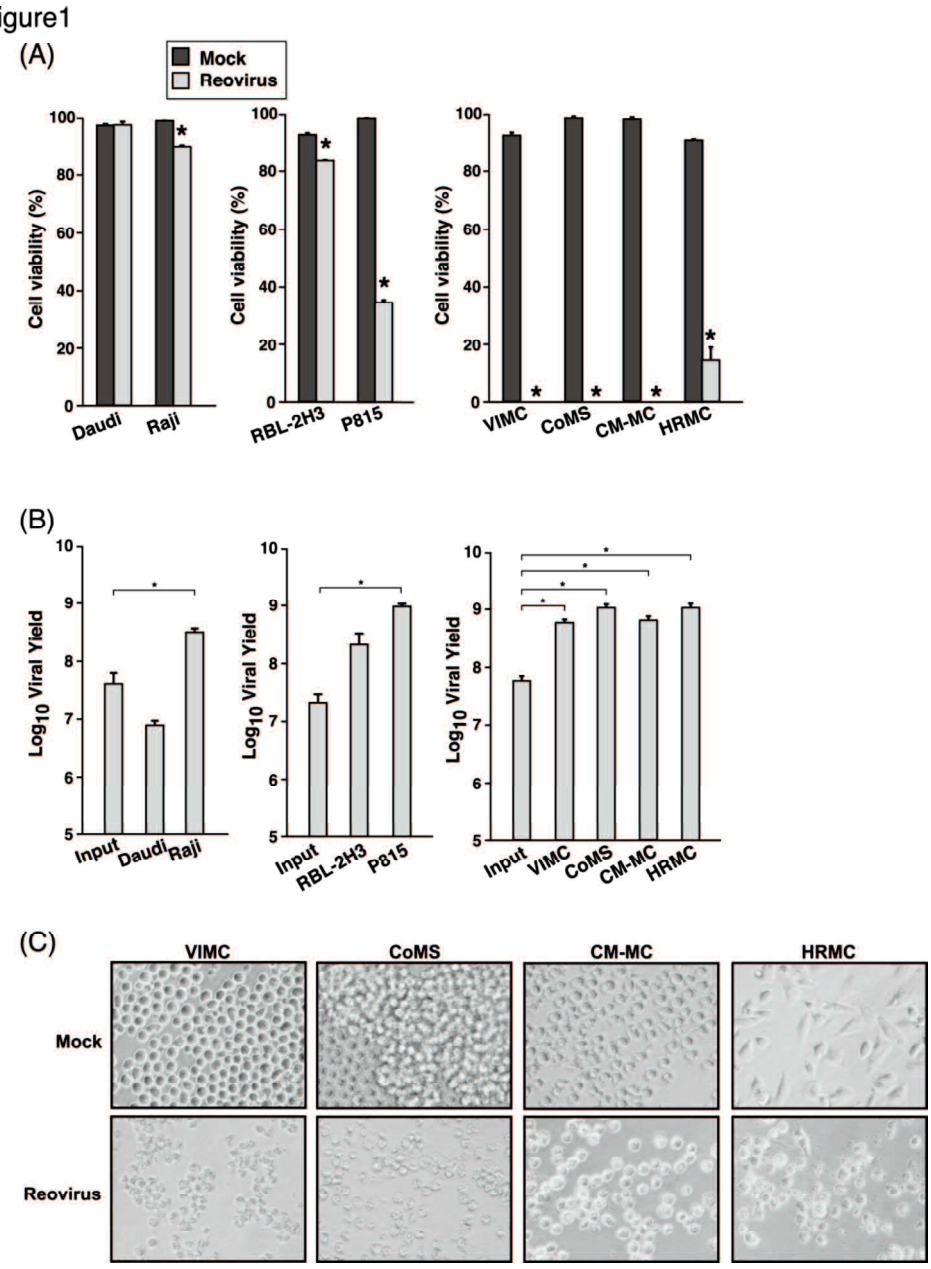


Fig. I-1. Reovirus effectively exerts effect in MCT cell lines. (A) VIMC, CoMS, CM-MC, HRMC, RBL-2H3 and P815 cells in triplicate wells were either mock-infected or infected with reovirus at MOI 70. Human Burkitt's lymphoma cell lines, Daudi and Raji, were used as negative and positive controls respectively. After 72 hours post-infection (hpi), cell viability was assessed with 0.25% trypan blue. Mean ± SD, n = 3, * $p<0.05$ (by Student's *t* test). (B) Supernatant of reovirus-infected (MOI 70) MCT cell lines was harvested at 72 hpi before input and progeny virus titer were determined by $TCID_{50}$ assay. Log_{10} mean viral yield ± SD, n = 3, * $p<0.05$ (by Student's *t* test). (C) Photomicrographs of mock-infected (upper panels) and reovirus-infected (MOI 70; lower panels) canine MCT cells taken at 72 hpi.

Figure 2

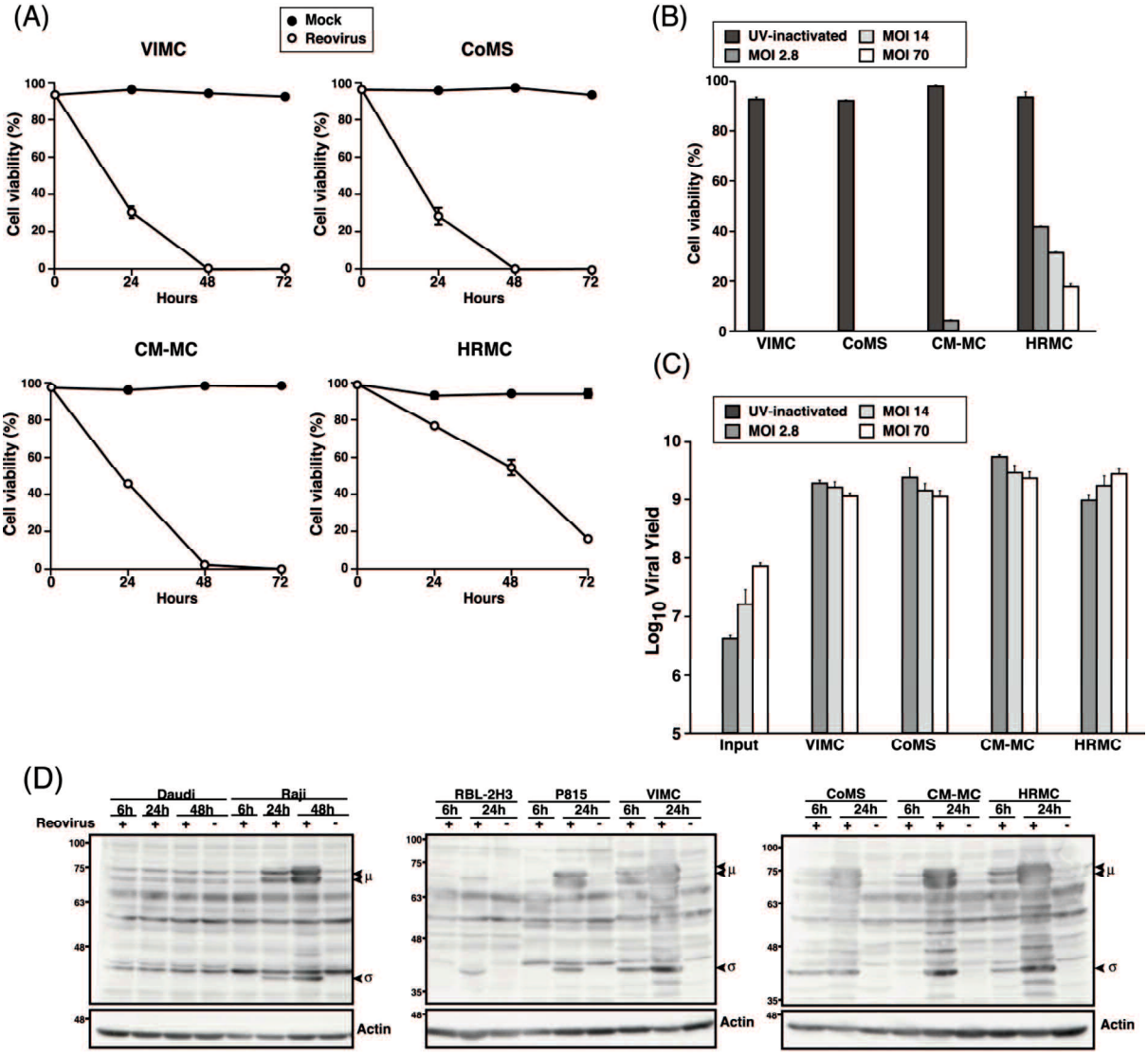


Fig. I-2. Reovirus infectivity and replication in MCT cell lines. (A) Cell viability of reovirus-infected (MOI 70) canine MCT cell lines were assessed with 0.25% trypan blue at 0, 24, 48 and 72 hpi. (B) Canine MCT cell lines were treated with UV-inactivated, 2.8, 14 and 70 MOI of reovirus and cell viability was assessed with 0.25% trypan blue at 72 hpi. (C) Input and progeny virus titer of supernatant from reovirus-infected MCT cell lines at various MOI were determined by $TCID_{50}$ assay. Mean ± SD, n = 3. (D) Whole cell lysates of reovirus-infected (MOI 70) of cell lines at 6 and 24 hpi (48 hpi only for Daudi and Raji) were prepared before proteins were separated using SDS-PAGE. Presence of μ and σ outer capsid proteins was determined using rabbit polyclonal anti-reovirus antibody. Actin was used as protein loading controls.

Figure 3

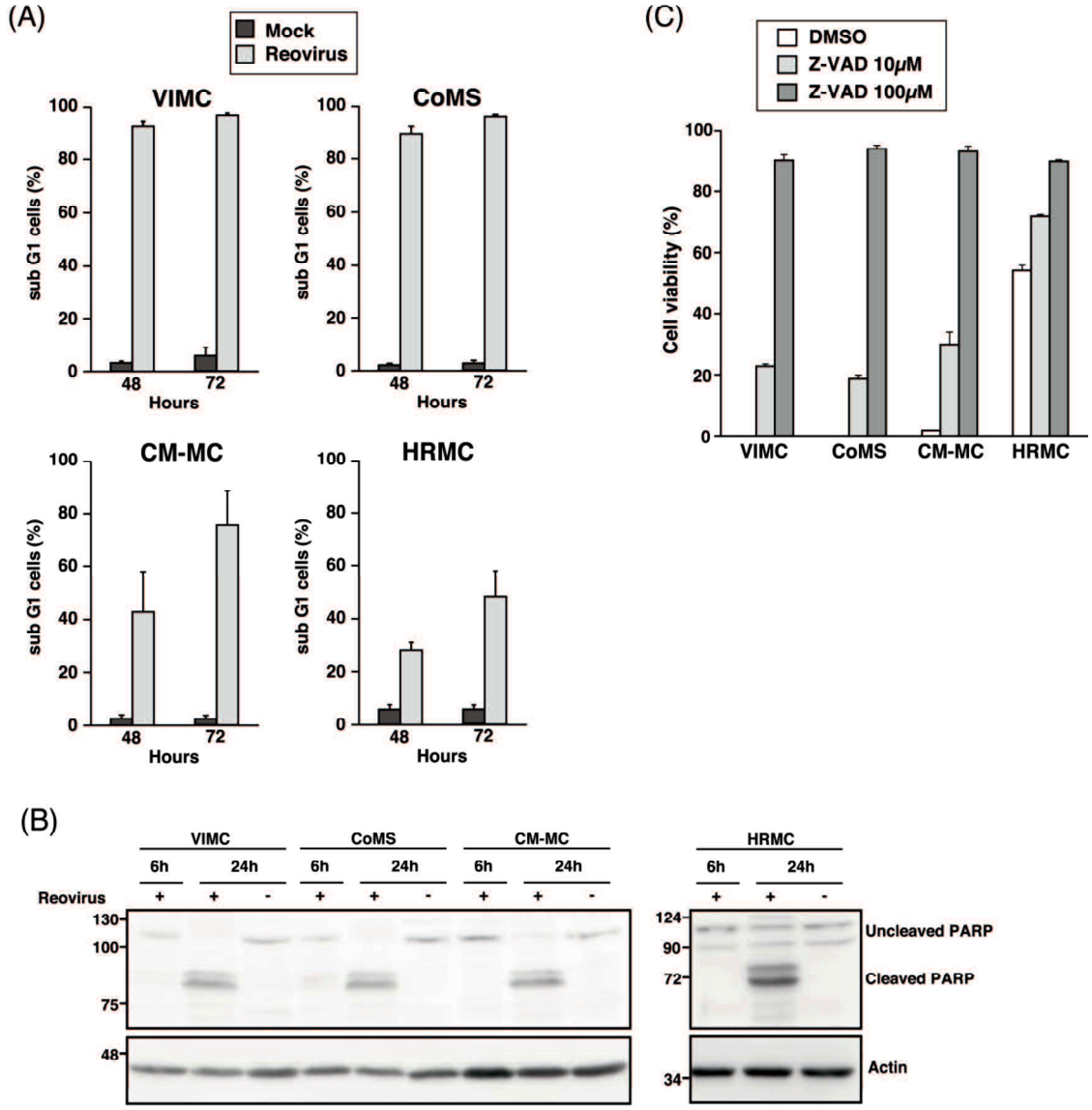


Fig. I-3. Reovirus induces cell death in MCT cell lines via the apoptosis pathway. (A) Reovirus-infected (MOI 70) canine MCT cell lines were harvested at 48 or 72 hpi and stained with propidium iodide (PI) before fluorescence-activated cell sorting (FACS) acquisition. Cell death was determined by the proportion of subG1 cells. Mean ± SD, n = 3. (B) Whole cell lysates of reovirus-infected (MOI 70) canine MCT cell lines at 6 and 24 hpi were prepared before proteins were separated using SDS-PAGE. Presence of Poly(ADP-Ribose) Polymerase (PARP) cleavage was determined using anti-PARP antibody. Actin was used as protein loading controls. (C) Canine MCT cell lines in triplicate wells were pre-treated with DMSO, 10 μM or 100 μM of Z-VAD-FMK for 30 minutes at 37˚C incubator before being infected with reovirus at MOI 70. Cell viability was assessed with 0.25% trypan blue at 48 hpi. Mean ± SD, n = 3.

Figure 4

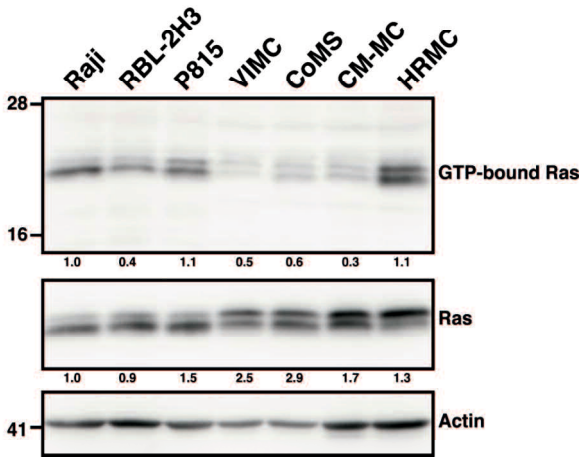


Fig. I-4. Ras activation does not correlate with reoviral susceptibility in MCT cell lines. GST-RBD protein was extracted and immobilized on glutathione-Sepharose beads to affinity-precipitated Ras-GTP from cell lysates. The affinity-precipitated Ras-GTP and whole cell lysates were subjected to SDS-PAGE before detection by Western blotting with anti-Ras antibody. The number below each lane represents the densitometry analysis of GTP-bound Ras relative to actin (upper row) and total Ras relative to actin (lower row) using ImageJ. Raji was used as the standard for Ras activation.

Figure 5

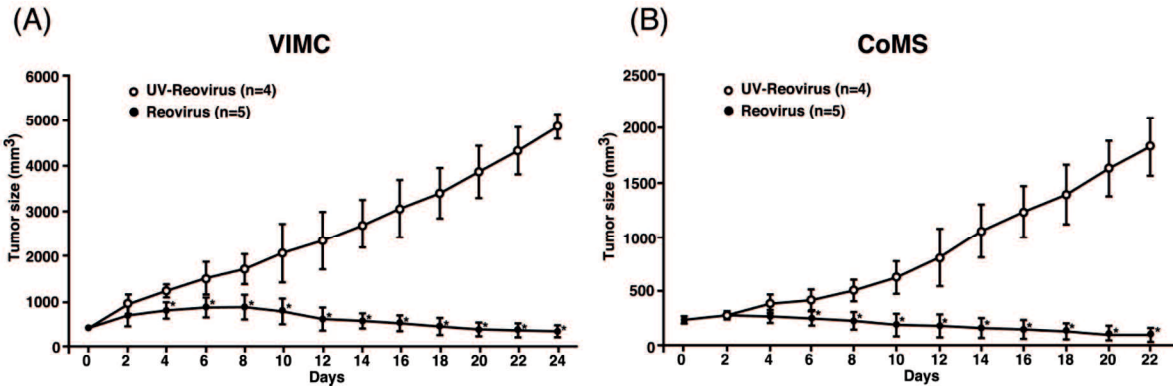


Fig. I-5. Reovirus effectively reduces tumor mass in unilateral MCT xenograft mouse models. (A) VIMC and (B) CoMS (1.0×10^7 cells) were implanted subcutaneously at the right flank. Once the desired tumor size was achieved (day 0), tumors were treated with a single intratumoral injection of 7.0×10^7 PFUs of reovirus or UV-inactivated reovirus. Tumor size was measured every other day with a caliper. Mean ± SD, n = 3, * $p<0.05$ (by Student's *t* test).

Figure 6

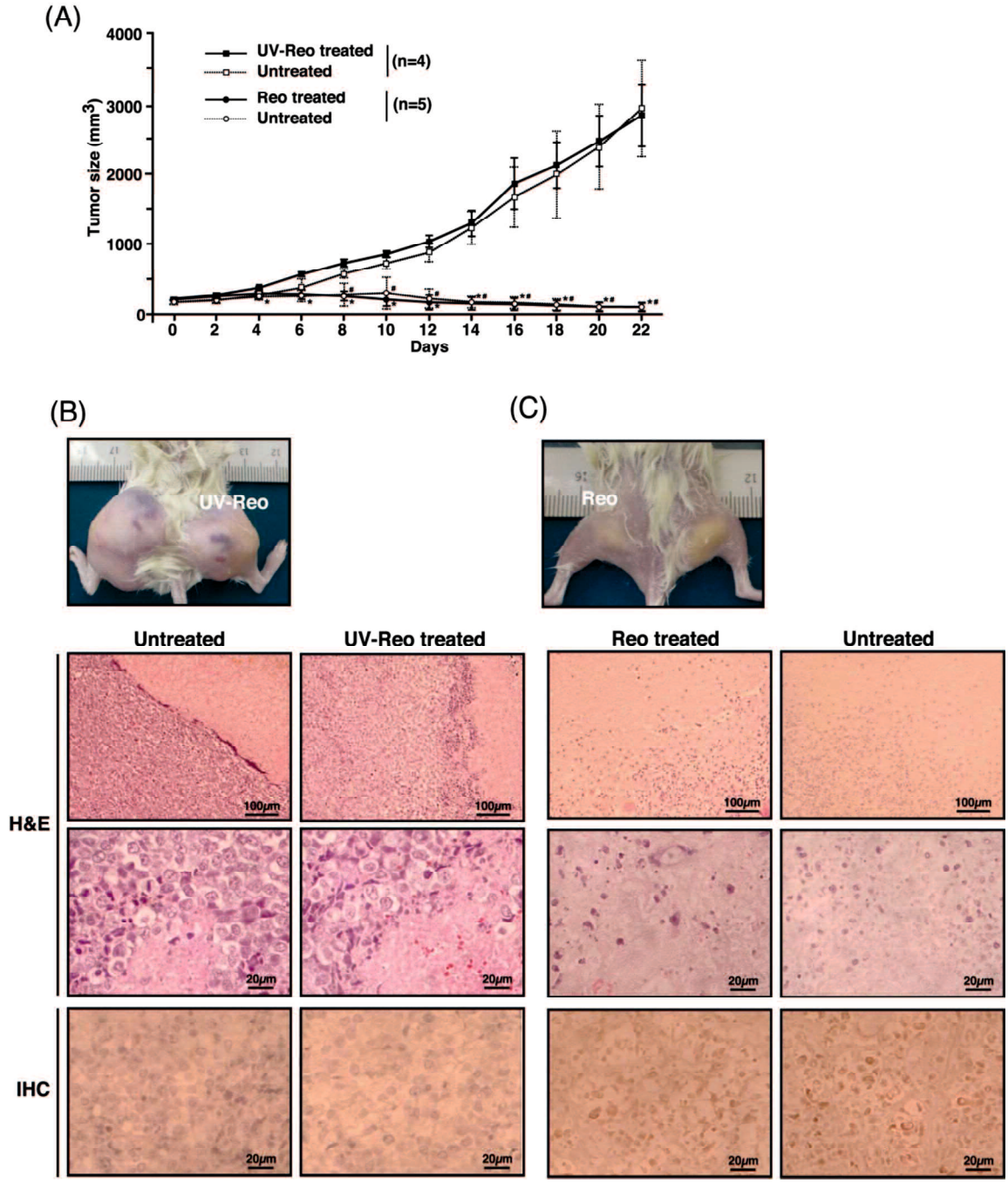


Fig. I-6. Gross and histological appearance of VIMC bilateral MCT xenograft mouse models. (A) VIMC (1.0 x 10^7 cells) was implanted subcutaneously into both flanks. When either one of the tumors reached the desired size (day 0), tumors were treated unilaterally with a single intratumoral injection of 7.0 x 10^7 PFUs of reovirus or UV-inactivated reovirus. Tumor size was measured every other day with a caliper. Mean ± SD, n = 3, * $p<0.05$ between reovirus and UV-inactivated reovirus treated masses; #, $p<0.05$ between the contralateral masses. Gross appearance, H&E and IHC histological samples of representative bilateral VIMC mice 22 days after treatment with UV-inactivated reovirus (B) or reovirus (C).

Figure 7

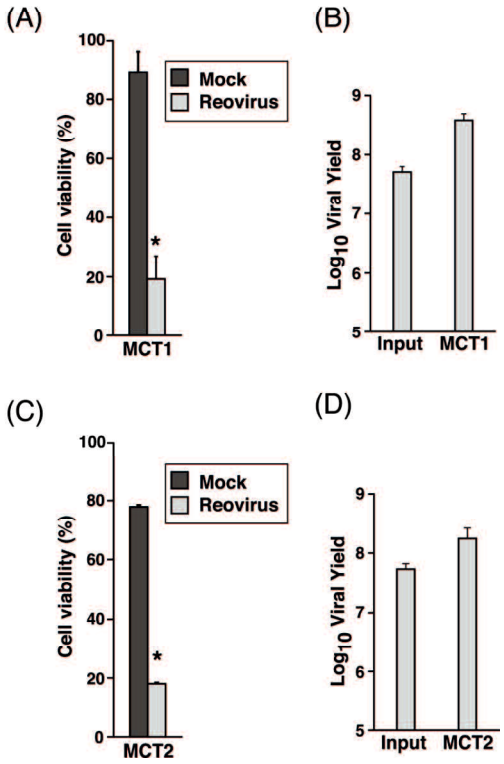


Fig. I-7. Reovirus induces significant cell death in primary canine MCT samples. Primary canine MCT samples in triplicate wells were either mock-infected or infected with reovirus at MOI 70. Cell viability was assessed with 0.25% trypan blue at 72 hpi before input and progeny virus titer were determined by $TCID_{50}$ assay. Mean ± SD, n = 3, MCT 1 (A, B). Mean ± SD, n = 2, MCT 2 (C, D). * $p<0.05$ (by Student's *t* test).

Figure 8

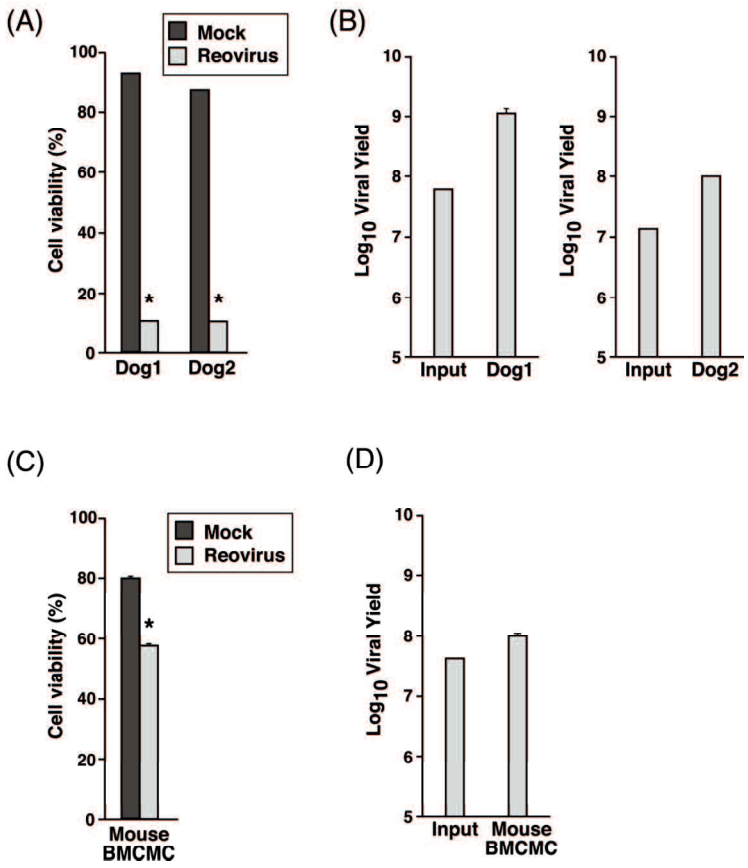


Fig. I-8. Reovirus induces dramatic effects in canine BMCMC but milder effects in murine BMCMC. Canine and murine BMCMC in triplicate wells were either mock-infected or infected with reovirus at MOI 70 before cell viability was examined with 0.25% trypan blue at 72 hpi before input and progeny virus titer were determined using $TCID_{50}$ assay. Mean ± SD, dog 1 and 2 (A, B). Mean ± SD, n = 3 (C, D). * $p<0.05$ (by Student's *t* test).

CHAPTER 2

Effects of reovirus in canine lymphoma *in vitro*

SUMMARY

In the previous chapter, canine mast cell tumor was proven to be susceptible to the oncolytic effects of reovirus. In order to further explore the potential of reovirus in veterinary oncology, studies have been undertaken to screen the susceptibility of other canine cancers to reovirus and determine the correlation to Ras activation. In this study, ten canine lymphoma cell lines were tested to determine the effects of reovirus. Reovirus-induced cell death, virus replication and infectivity were confirmed in four of the cell lines with variable levels of susceptibility. All the cell lines that were susceptible to reovirus underwent apoptosis as proven by propidium iodide (PI) staining, cleavage of Poly (ADP-Ribose) Polymerase (PARP) and inhibition of cell death by caspase inhibitor. The level of Ras activation varied among the cell lines with no correlation with the susceptibility to reovirus. Unlike canine MCT, the effects of reovirus as monotherapy in canine lymphoma cell lines are limited. Hence, combination therapy using reovirus and chemotherapy in these cell lines is currently under investigation.

INTRODUCTION

Lymphoma originates from lymphoreticular cells, and usually arises in lymphoid tissues, such as the spleen, lymph nodes and bone marrow. Canine lymphoma is the most common canine hematopoietic tumor, making up of 83% of all canine hematopoietic malignancies and 7 to 24% of all canine neoplasms (Vail et al., 2013). Since lymphoma is considered a systemic disease, systemic therapies, especially multiagent chemotherapeutic approaches, are the best available options, with response rates up to 90%. Unfortunately, most animals will eventually succumb to relapse due to chemotherapy resistance (Marconato, 2011). Alternative therapies using monoclonal antibody and anti-tumor vaccine have been reported but effects were limited (Vail et al., 2013). Among the studies of oncolytic virotherapy in veterinary oncology, only the canine distemper virus (CDV) has been tested in canine lymphoid cells (Suter et al., 2005). Preclinical data from this study were encouraging, where CDV was reported to be a feasible therapeutic option in canine lymphoma.

On the other hand, the effects of reovirus have been tested in various human lymphoid malignancies with variable results (Alain et al., 2002; Thirukkumaran et al., 2003; Alain et al., 2010). Due to the similarities of canine lymphoma and the human variant of non-Hodgkin's lymphoma (NHL) (Fournel-Fleury et al., 1997), we hypothesize that reovirus can serve as an alternative treatment in canine lymphoma. In this chapter, the effects of reovirus in ten established canine lymphoma cell lines were evaluated, followed by the determination of the cell death pathway. In addition, the Ras activation status of each cell line was examined in hope to elucidate the role of the Ras signaling pathway in reovirus-induced cell death in canine lymphoma.

MATERIALS AND METHODS

Cell cultures and reovirus

A total of ten established canine lymphoma cell lines were tested for their susceptibility to reovirus. CL-1 (Momoi et al., 1997), UL-1 (Yamazaki et al., 2008), CLGL-90 (Suter et al., 2005), Nody-1, Ema and CLK (Umeki et al., 2012) are T cell lymphoma cell lines while GL-1 (Nakaichi et al., 1996), 17-71 (Steplewski et al., 1987) and CLBL-1 (Rütgen et al., 2010) are B cell lymphoma cell lines. CLC is a canine lymphoma cell line established in our laboratory but the cell type could not be determined (Umeki et al., 2012). Mouse L929 fibroblastic cell line and human Burkitt's lymphoma cell line, Raji were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University). All cell lines, except for 17-71 and CLGL-90, were maintained in R10 complete medium (RPMI1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 55 μM 2-mercaptoethanol). 17-71 and CLGL-90 were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 4.4 mM L-glutamine. All cells were grown at 37˚C in a humidified 5% CO_2 incubator.

The Dearing strain of reovirus serotype 3 (Reolysin; clinical grade reovirus; GMP) was obtained from Oncolytics Biotech Inc.. Ultraviolet (UV)-inactivated virus was prepared by exposing live virus to UV light for 90 minutes.

Reovirus infection of cell lines

CL-1, 17-71, GL-1, Ema, Nody-1 and CLK were seeded at 2.5 x 10^4 cells; UL-1 and CLBL-1 were seeded at 1.25 x 10^4 cells; CLC and CLGL-90 were seeded at 3.125 x 10^3 cells before being mock-infected or infected with reovirus at multiplicity of infection (MOI) of 70 plaque-forming units (PFUs) per cell. Cell infection was performed in triplicates in 48-well

plates. Cell viability was quantified by trypan blue exclusion test at 72 hours post-infection (hpi). Supernatant from each sample was collected and kept at -80 ˚C, pending titration of progeny virus using 50% tissue culture infectious dose ($TCID_{50}$) assay on L929 cells, as previously described (Reed and Muench, 1938) with modifications.

Cell lines susceptible to reovirus (CL-1, 17-71, CLC and GL-1) were selected in the assessment of cell viability curves at 0, 24, 48 and 72 hpi at MOI 70 of reovirus. In order to investigate the susceptibility towards various titre of reovirus, only the two most susceptible cell lines were selected and incubated with UV-inactivated, 2.8, 14 and 70 MOI of reovirus for 72 hours.

Western blotting

All reovirus-susceptible canine lymphoma cell lines and two reovirus-resistant cell lines (Nody-1 and CLGL-90) were selected to determine the infectivity of reovirus. These cells were seeded at 5.0×10^5 and mock-infected or infected with reovirus at MOI 70 for 48 hours. Whole cell lysates were treated with RIPA lysis buffer (50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate), supplemented with complete, Mini EDTA-free protease inhibitor mixture (Roche Diagnostics K.K., Tokyo, Japan).

As for PARP cleavage detection, only CL-1, 17-71 and CLC were prepared in the same manner and harvested at 6 and 48 hpi. Cell lysates were lysed with NP40 lysis buffer (1% NP40, 10 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA), also supplemented with complete, Mini EDTA-free protease inhibitor mixture.

Proteins for reovirus infectivity and PARP cleavage were subjected to SDS-PAGE and Western blotting. Primary antibodies were rabbit anti-reovirus (produced by our lab), rabbit anti-PARP (NeoMarkers) and secondary antibody goat anti-rabbit IgG HRP (Zymed Laboratories). Goat anti-actin (Santa Cruz Biotechnology) and rabbit anti-goat IgG HRP

(Bethyl Laboratories) were used as loading controls.

Propidium iodide (PI) staining

CL-1, 17-71 and GL-1 were seeded at 1.0 x 10^5 while CLC was seeded at 1.25 x 10^4 before being infected with reovirus at MOI 70 and cells were harvested at 48 and 72 hpi. Cell treatment and PI staining were subsequently performed as previously described in Chapter 1.

Inhibition of reovirus killing by Z-VAD-FMK

CL-1 and 17-71 were seeded at 2.5 x 10^4 cells while CLC was seeded at 3.125 x 10^3 cells and pre-treated with control DMSO, 10 μM or 100 μM of Z-VAD-FMK (caspase inhibitor I; Calbiochem) for 30 minutes at 37˚C before being infected with reovirus at MOI 70. Cell viability was quantified by trypan blue exclusion test at 48 hpi.

GST pull-down assay for Ras status

Ras activation status of all the canine lymphoma cell lines was evaluated after GST pull-down according to Chapter 1. Mouse anti-pan-Ras (Calbiochem) and goat anti-mouse IgG HRP (Zymed Laboratories) were used as primary and secondary antibodies respectively.

RESULTS

Susceptibility of canine lymphoma cell lines to reovirus-induced cell death

In order to test the susceptibility of canine lymphoma towards reovirus infection, a total of ten established canine lymphoma cell lines were screened by infection of reovirus at MOI 70 before assessment of cell viability at 72 hpi. Trypan blue exclusion test revealed the direct reovirus-induced cell death in four out of ten cell lines (Fig. II-1A; $P<0.05$). Next, to determine if reovirus-susceptible cell lines can sustain the infection, the amount of progeny virus produced was measured by $TCID_{50}$ assay. Correlatively, only the cell lines that displayed a reduction in cell viability had significant increment of progeny virus as compared to the input virus titre (Fig. II-1B; $P<0.05$). Morphological characteristics of cytopathic effects in the reovirus-susceptible cell lines such as granular appearance, cell clumping and loss of shape were shown in Fig. II-1C. Among the cell lines that were susceptible to reovirus, one was T cell lymphoma (CL-1), two were B cell lymphoma (17-71 and GL-1) and the cell type of CLC was undetermined.

Reovirus infectivity varies in canine lymphoma cell lines

At 24 hpi, reovirus induced cell death at a comparable rate in CL-1, 17-71 and GL-1 but not in CLC (Fig. II-2A). However, the rate of cell death accelerated at 48 and 72 hpi for CL-1 and 17-71 as compared to GL-1 and CLC. In Fig. II-2B, the viability of the two most susceptible cell lines was compared using reovirus at various MOI of 2.8, 14 and 70. Unlike in 17-71, CL-1 had a higher susceptibility to reovirus at a low MOI, suggesting that there exists a difference in the sensitivity towards reovirus between the cell lines. In order to confirm that cell death was induced by reovirus, SDS-PAGE and Western blotting were carried to detect the reovirus μ and σ proteins. By 48 hpi, the μ and σ proteins were detected only in the reovirus-susceptible cell lines but not in the two representatives of the reovirus-

resistant cell lines, Nody-1 and CLGL-90 (Fig. II-2C).

Reovirus induces canine lymphoma cell death via apoptosis

Previous reports have indicated that reovirus induces apoptosis in susceptible cell lines (Connolly et al., 2000; Clarke et al., 2004). Therefore, in this study, the apoptotic cell death due to reovirus was investigated using PI staining, detection of PARP cleavage and treatment of Z-VAD-FMK. As expected, the percentage of PI-stained cells increased from 48 to 72 hpi in all the reovirus-susceptible cell lines (Fig. II-3A). The cleavage of cellular substrate PARP is the morphological hallmark of apoptosis. Signature cleavage product in CL-1, 17-71 and CLC was not detectable at an early time point but became obvious at 48 hpi (Fig. II-3B). On top of that, pre-treatment of canine lymphoma cell lines with Z-VAD-FMK before reovirus infection allowed the inhibition of reovirus-induced apoptosis in a dose-dependent manner (Fig. II-3C).

Ras activation status does not correlate with reoviral susceptibility

The baseline GTP-loading status of Ras was determined for the ten canine lymphoma cell lines in order to investigate the involvement of Ras activation as the molecular determinant for reoviral susceptibility. Using Raji as the standard of Ras activation, GL-1, Ema, Nody-1, CLGL-90, CLBL-1, CLC and CLK had elevated Ras activities while Ras was not activated in CL-1, UL-1 and 17-71 (Fig. II-4). Comparison of the susceptibility of the cell lines to reovirus with their activated Ras status revealed that even though CL-1 and 17-71 were highly susceptible to reovirus, they did not express higher GTP-bound Ras levels. This finding indicates that mechanisms other than the activation of Ras are involved in the attribution of reoviral susceptibility.

DISCUSSION

This chapter highlights the cytotoxic effects of reovirus in canine lymphoma cell lines. At first, a panel of ten established canine lymphoma cell lines was screened to assess their susceptibility to reovirus. Out of these cell lines, six were T cell lymphoma, three were B cell lymphoma and one cell line with an undetermined cell type. Four of the cell lines were susceptible to the effects of reovirus as indicated by reovirus-induced cytotoxicity (Fig. II-1A), viral progeny production (Fig. II-1B) and reovirus infectivity (Fig. II-2C). However, there was no distinguishable pattern as to which cell type has a higher susceptibility to reovirus (Table II-1).

Similar studies of the effects of reovirus in a variety of human NHL have also been performed. The types of human NHL that were involved are diffused large B-cell lymphoma, Burkitt lymphoma, chronic lymphocytic leukemia, Mantle cell lymphoma, small lymphocytic lymphoma, Follicular lymphoma and Waldenström macroglobulinemia. However, the susceptibility of these neoplasms towards reovirus ranges markedly, from sensitive to resistant (Alain et al., 2002; Thirukkumaran et al., 2003; Alain et al., 2010). Similarly, the results of this study using canine lymphoma cell lines revealed noticeable differences in the susceptibility to reovirus.

CL-1 and 17-71 were highly susceptible to reovirus (Fig. II-2A and II-3A) but CL-1 proved to be more sensitive at a lower MOI (Fig. II-2B). The susceptible cell lines underwent apoptotic cell death in a caspase-dependent manner after reovirus infection (Fig. II-3), which is consistent with previous report (Marcato et al., 2007). Reovirus-induced apoptosis takes place slower in canine lymphoma cell lines as compared to canine MCT, where the cleavage of PARP was detected as early as 6 hpi (Chapter 1, Fig. I-3B). Besides apoptosis, reovirus infection also leads to necrosis (Ikeda et al., 2004), necroptosis (Berger and Danthi, 2013) and autophagy (Thirukkumaran et al., 2013). Combination of these findings strongly

suggests that reovirus deploys different cell death pathway depending on the type of cells involved. As such, this emphasises the need for additional studies to find out how reovirus utilises these cell death pathways in order to pinpoint the exact mechanism of reovirus oncolysis.

The initial studies on the mechanism of reovirus-induced cell death suggested that Ras transformation in cells promotes three reoviral replication steps, which are proteolytic disassembly, viral infectivity and efficient reovirus release (Marcato et al., 2007). However, subsequent studies have shown that the situation is highly complex, and no definitive biomarker of sensitivity to reovirus has been identified as of yet (Sahin et al., 2013). In this study, the level of activated Ras in the canine lymphoma cell lines did not correlate to reoviral susceptibility (Fig. II-4; Table II-1). This finding is similar to that obtained in another study using canine mast cell tumor that was reported in Chapter 1. Indeed, it can't be denied that there exists a possibility that the mechanism of reovirus-induced cell death is different in human and canine cancers. Therefore, further studies are being carried out to elucidate the mechanism of reovirus-induced cell death in canine cancers.

Reovirus serves as an attractive option for cancer therapy. As the wild type reovirus is naturally cytotoxic to cancer cells, the utilization of reovirus seems to be superior to the other genetically engineered oncolytic viruses. The minimal side effects that were reported in human clinical trials provide further reassurance of the safety of administration of reovirus. On top of that, the life cycle of reovirus takes place in the cytoplasm and does not include a stage of viral DNA synthesis (Schiff et al., 2007). Therefore, it is very unlikely that reoviral genome fragments would integrate into the DNA and cause harmful mutations. Even with all these advantages, reovirus as a monotherapy still has limited therapeutic efficacy, especially in clinical settings. A different approach with combinations of reovirus and conventional (chemo- or radiation) therapies has been carried out in multiple phase I, II and III clinical

studies (Sahin et al., 2013). Therefore, in order to increase the oncolytic effects of reovirus in canine cancers, concomitant usage of reovirus and chemotherapeutic agent(s) sounds like an attractive option that still requires additional studies.

In summary, the susceptibility of canine lymphoma cell lines to the effects of reovirus varied considerably. The reovirus-susceptible cell lines underwent apoptotic cell death, which correlated with the production of progeny virus and detection of viral proteins. However, Ras activation did not correlate with reoviral susceptibility in the cell lines. Further analysis of the mechanism of reovirus-induced cell death is ongoing. In order to improve the therapeutic efficacy of reovirus in canine lymphoma, combination studies using reovirus and chemotherapeutic agents should be tested *in vitro*.

TABLE

Table II-1. Summary of the effects of reovirus in ten established canine lymphoma cell lines.

Cell line	Cell type	Reoviral susceptibility[a]	Viral progeny production[b]	Ras activation
CL-1	T cell	+++	+++	-
17-71	B cell	+++	+++	-
CLC	Undetermined	++	+++	+
GL-1	B cell	+	+	+
Ema	T cell	-	-	+
UL-1	T cell	-	-	-
Nody-1	T cell	-	-	+
CLGL-90	T cell	-	-	+
CLBL-1	B cell	-	-	+
CLK	T cell	-	-	+

[a] Reoviral susceptibility: - No significant difference between cell viability of mock and reovirus-infected cells; + <20% cell death; ++ 20-50% cell death; +++ >50% cell death.

[b] Viral progeny production: - No significant increment as compared to input virus titre, + <5x increment of virus titre; ++ 5-10x increment of virus titre; +++ >10x increment of virus titre.

FIGURES

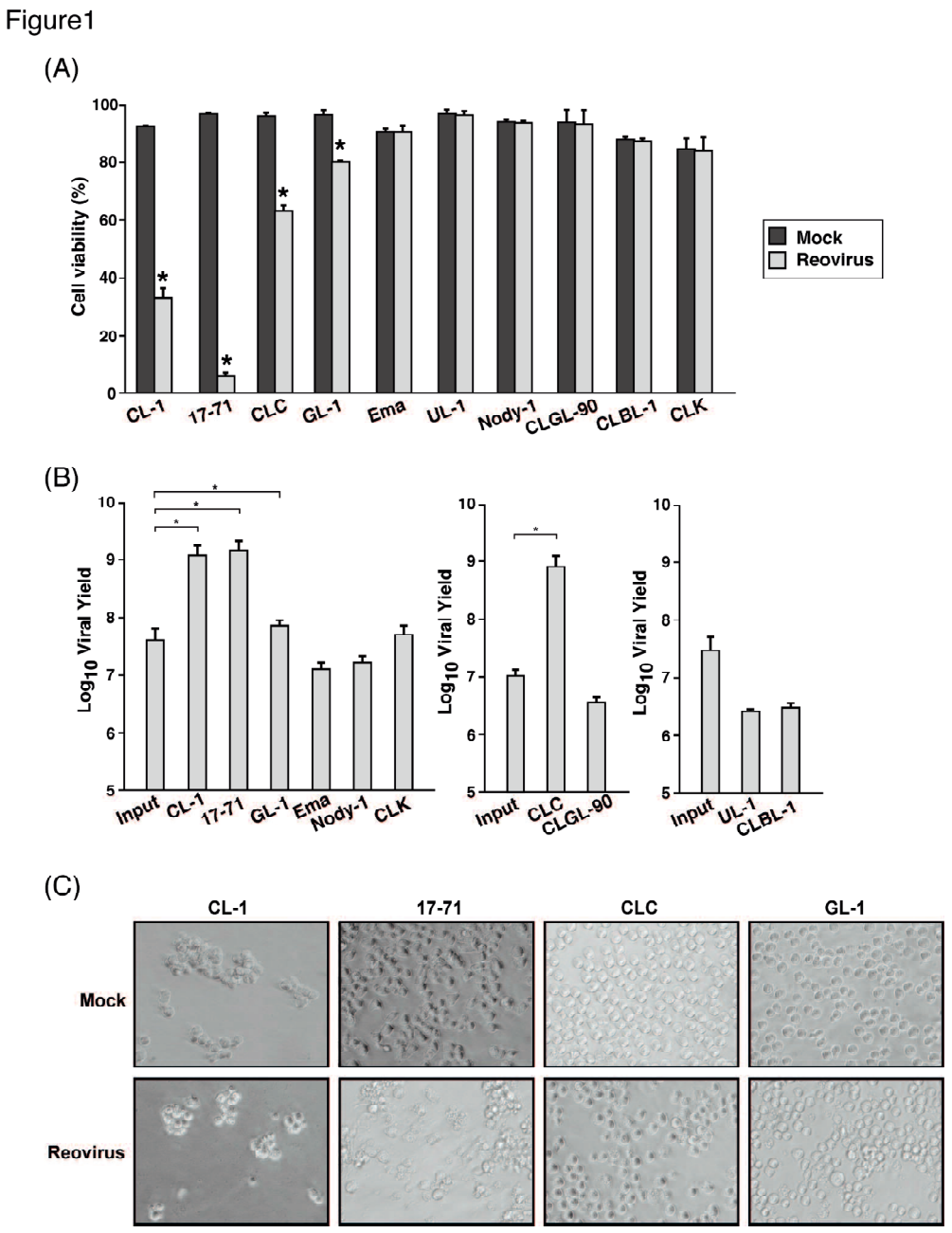


Fig. II-1. Effects of reovirus in canine lymphoma cell lines. (A) Ten canine lymphoma cell lines in triplicate wells were either mock-infected or infected with reovirus at MOI 70. After 72 h post-infection (hpi), cell viability was quantified by trypan blue exclusion test. Mean ± SD, n = 3, * $p<0.05$. (B) Supernatant of reovirus-infected (MOI 70) canine lymphoma cell lines was harvested at 72 hpi before input and progeny virus titre were determined by $TCID_{50}$ assay. Mean ± SD, n = 3, * $p<0.05$. (C) Photomicrographs of reovirus-susceptible canine lymphoma cell lines taken at 72 hpi. Upper panels: mock infection, lower panels: reovirus infection at MOI 70.

Figure 2

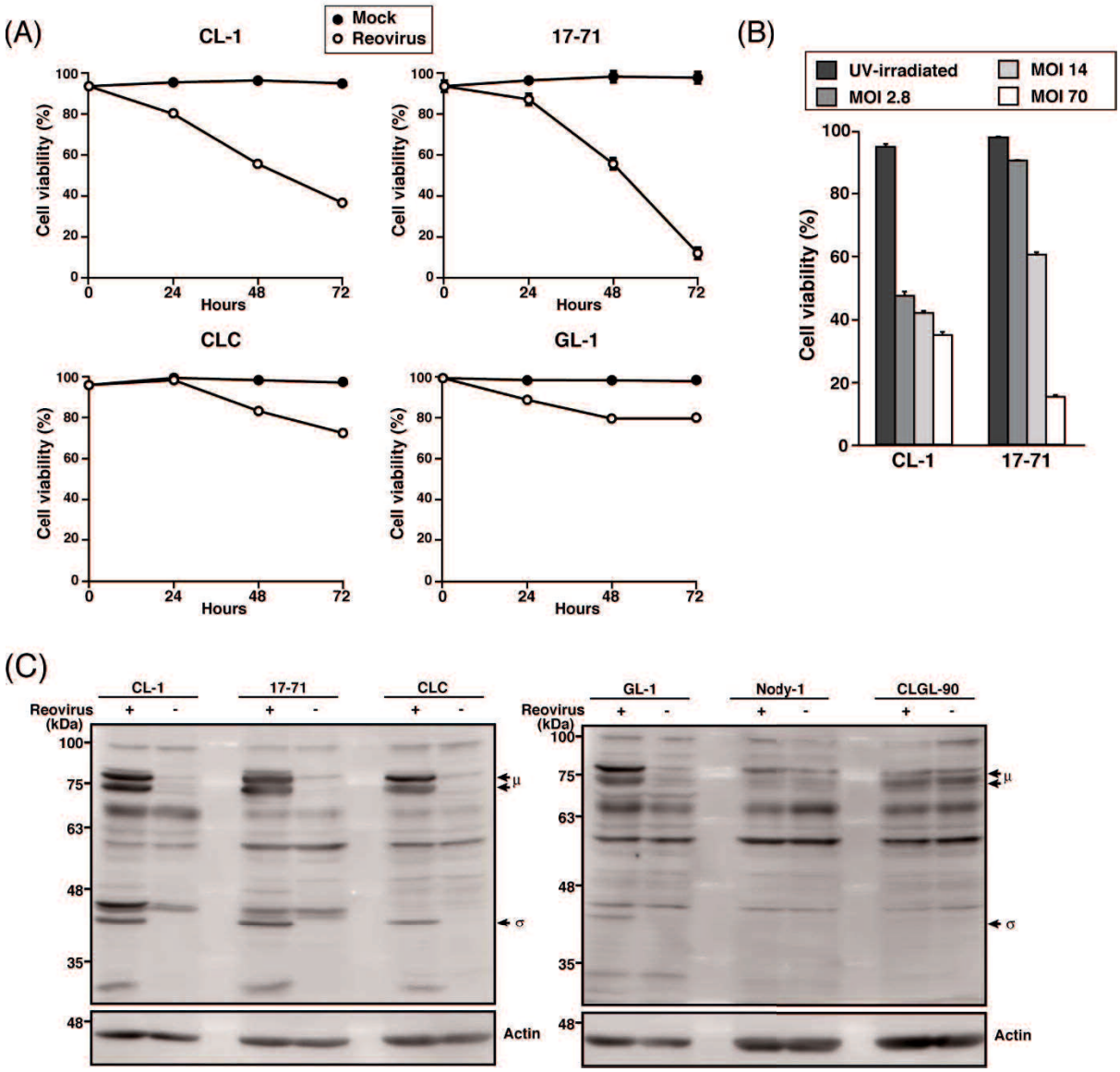


Fig. II-2. Reovirus infectivity and replication in reovirus-susceptible canine lymphoma cell lines. (A) Cell viability of reovirus-infected (MOI 70) CL-1, 17-71, CLC and GL-1 was quantified by trypan blue exclusion test at 0, 24, 48 and 72 hpi. Mean ± SD, n = 3. (B) CL-1 and 17-71 were treated with UV-inactivated, 2.8, 14 and 70 MOI of reovirus and cell viability was quantified by trypan blue exclusion test at 72 hpi. Mean ± SD, n = 3. (C) All reovirus-susceptible and two representatives of reovirus-resistant (Nody-1 and CLGL-90) canine lymphoma cell lines were either mock-infected or infected with reovirus at MOI 70. Cells were collected at 48 hpi and whole cell lysates were prepared before proteins were separated using SDS-PAGE. Presence of μ and σ outer capsid proteins was determined using rabbit polyclonal anti-reovirus antibody. Actin was used as protein loading controls.

Figure 3

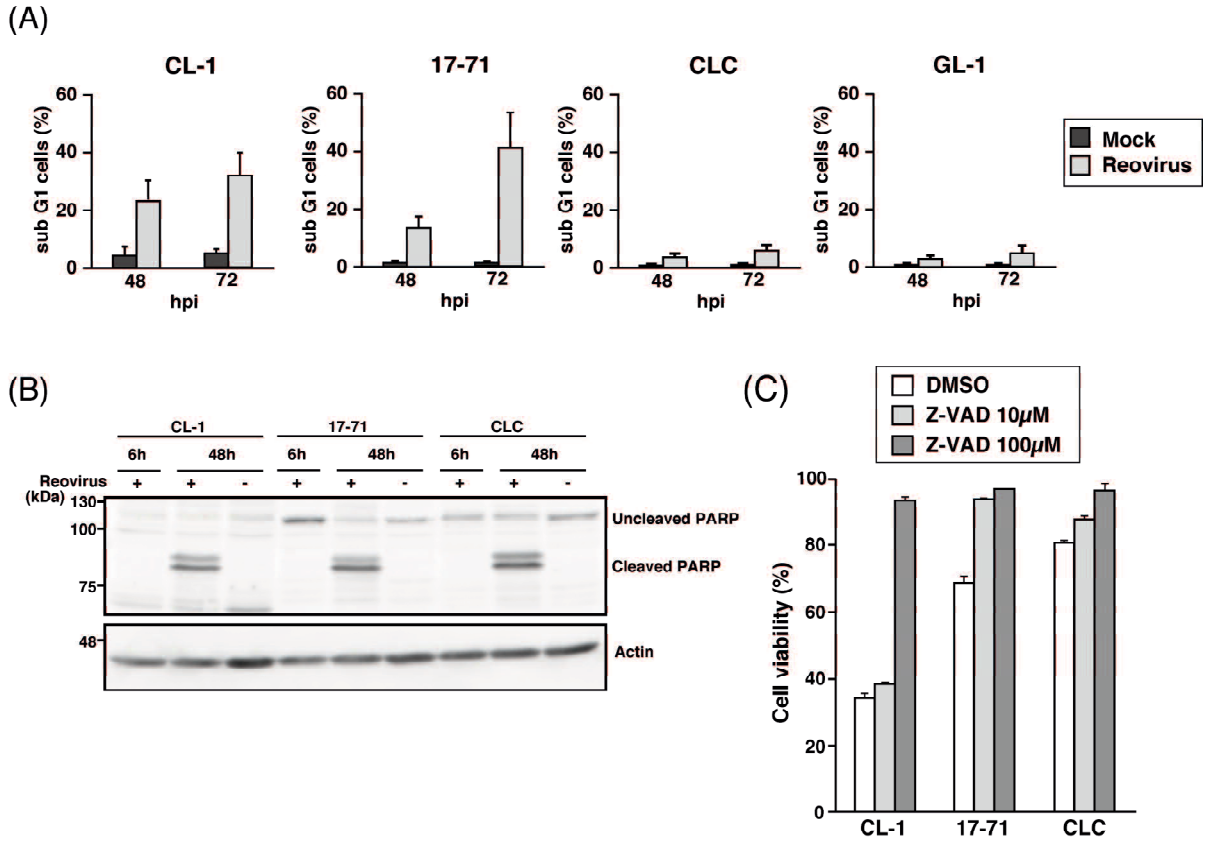


Fig. II-3. Reovirus induces cell death in canine lymphoma cell lines via the apoptosis pathway. (A) Reovirus-infected (MOI 70) CL-1, 17-71, CLC and GL-1 were harvested at 48 or 72 hpi and stained with propidium iodide (PI) before fluorescence-activated cell sorting (FACS) acquisition. Cell death was determined by the proportion of sub G1 cells. Mean ± SD, n = 3. (B) Whole cell lysates of reovirus-infected (MOI 70) CL-1, 17-71 and CLC at 6 and 48 hpi were prepared before proteins were separated using SDS-PAGE. Presence of Poly (ADP-Ribose) Polymerase (PARP) cleavage was determined using anti-PARP antibody. Actin was used as protein loading controls. (C) CL-1, 17-71 and CLC in triplicate wells were pre-treated with DMSO, 10 μM or 100 μM of Z-VAD-FMK for 30 minutes at 37˚C incubator before being infected with reovirus at MOI 70. Cell viability was quantified by trypan blue exclusion test at 48 hpi. Mean ± SD, n = 3.

Figure 4

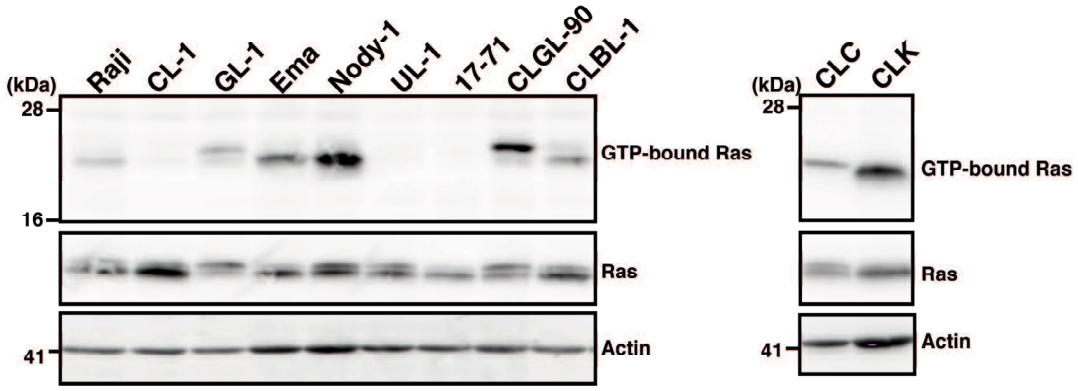


Fig. II-4. Ras activation does not correlate with reoviral susceptibility in canine lymphoma cell lines. GST-RBD protein was extracted and immobilised on glutathione-Sepharose beads to affinity-precipitated Ras-GTP from cell lysates. The affinity-precipitated Ras-GTP and whole cell lysates were subjected to SDS-PAGE before detection by Western blotting with anti-Ras Ab. Raji was used as the standard for Ras activation.

CHAPTER 3

Reovirus induces apoptotic cell death by the dysregulation of the Ras signaling pathway

SUMMARY

Although reovirus has reached phase II and III clinical trials in human cancers, the exact mechanism of reovirus oncolysis is still not completely understood. Various studies have indicated that the Ras activation status is not the sole determinant of the permissiveness of cells to reovirus. Nonetheless, it has been proven that Ras transformation enhances reovirus disassembly, infectivity and release of progeny virus. We have shown in Chapter 1 that canine mast cell tumor (MCT) cell lines are highly susceptible to reovirus, with VIMC and CoMS being the most susceptible, followed by CM-MC and HRMC. However, the susceptibility of the cell lines to reovirus does not have any correlation to the Ras activation status, which highlighted a need to clarify the role of the Ras signaling pathway in reovirus oncolysis. From the GST pull-down assay, the level of Ras-GTP decreased in all the cell lines after reovirus infection. On top of that, phosphorylation of all the downstream effectors of Ras (Raf, MEK, and ERK) and Akt was reduced in all the cell lines after reovirus infection, except for Akt in HRMC. The reovirus-induced apoptosis in these cell lines also relies on pro-apoptotic and anti-apoptotic proteins such as Bim, Bad and Mcl-1. Additionally, reovirus infection not only led to the dephosphorylation, but also degradation of c-Kit in all the cell lines. In short, reovirus infection dysregulates the Ras signaling pathway and degraded c-Kit in all the canine MCT cell lines, leading to apoptotic cell death. However, the difference of the susceptibility towards reovirus relies on its ability to downregulate the PI3K/Akt pathway.

INTRODUCTION

Epidermal growth factor receptor (EGFR) is a cell surface receptor belonging to the type I receptor tyrosine kinase family or erb-B family (Voldborg et al., 1997). Expressed primarily in cells of epithelial origin, EGFR signaling has been shown to be important not only for proliferation but also for other processes that are crucial in cancer progression, including angiogenesis, metastatic spread and the inhibition of apoptosis (Olayioye et al., 2000). The activation of EGFR phosphorylates a number of intracellular substrates including phospholipase C-gamma, (PLC-γ) and SHP-2 (Agazie and Hayman, 2003; Margolis et al., 1990). These effectors of receptor tyrosine kinase can potentially lead to catalytical activities activated by Ras (Dance et al., 2008; Kim et al., 2000; Oh-hora et al. 2003). Ras is a small GTP-binding protein, which is also a common upstream molecule of several signaling pathways including Raf/MEK/ERK and phosphoinositide 3-kinase (PI3K)/Akt (Peyssonnaux et al., 2000). Both these signaling cascades play critical roles in regulating gene expression, cell growth and tumorigenesis (McCubrey et al., 2006). Apoptosis can also be controlled by these pathways through the downregulation of ERK and Akt, targeting downstream proteins such as Mcl-1, Bim and Bad (Steelman et al., 2011).

Evidence of the differences in reoviral susceptibility in cells was first discovered when functional EGFR transfected into cells with no endogenous EGFR were enhanced in reovirus infectivity and viral progeny production (Strong et al., 1993; Strong et al., 1996). This suggests that reovirus takes advantage of one of the EGFR-related cell signaling pathways that has already been activated in the transformed cells. The activation of EGFR signaling triggers many downstream events, which might include phosphorylation of proteins, intracellular ion levels and the regulation of transcription and translation.

NIH-3T3 cells are resistant to reovirus and transformation with EGFR, Sos and Ras has elucidated the importance of the Ras signaling pathway in reoviral susceptibility (Strong et

al., 1998). The inhibition of viral mRNA translation was also reversed in NIH-3T3 cells transformed with the intermediates of the Ras signaling pathway. At the same time, it was also discovered that the phosphorylation of the double-stranded RNA-activated protein kinase (PKR) prevents viral synthesis in normal cells. However, PKR remained unphosphorylated in the transformed cells after reovirus infection. This report marks the beginning of a series of studies that has successfully proved that an activated Ras signaling pathway is required for effective reovirus oncolysis (Coffey et al., 1998; Marcato et al., 2007; Norman et al., 2004). Ras transformation enhances the disassembly of incoming virus, the infectivity and release of viral progeny from the infected cells (Marcato et al., 2007). These three crucial steps alone can increase the amplification of more than 100 fold of reovirus in the Ras-transformed cells.

We have shown in Chapter 1 and 2 that reovirus can induce cell death in all canine mast cell tumor (MCT) cell lines but the cytotoxic effects of reovirus on canine lymphoma cell lines was not as dramatic as expected. At the same time, we also found out that there was no correlation between reoviral susceptibility and the Ras activation status. However, we were unable to rule out the possibility of reovirus utilizing the existing Ras signaling pathway to exert its cytotoxic effects. Therefore, the objective of this chapter was to investigate the effects of reovirus on the Ras signaling pathway in canine MCT cell lines. Besides that, we also aimed to discover the involvement of other protein(s)/ pathway(s) that draws the line between cell lines that are highly susceptible and less susceptible to reovirus. Using four canine MCT cell lines, we investigated the effects of reovirus on: 1) GTP-bound Ras and total Ras, 2) Phosphorylation of Raf, MEK, ERK (Ras/Raf/MEK/ERK pathway), 3) Phosphorylation of Akt (PI3K/Akt pathway), 4) Bim, phosphorylation of Bad and Mcl-1 (apoptotic proteins downstream to ERK), 5) Phosphorylation of c-Kit, PLCγ1, PLCγ2 and SHP-2 (proteins upstream of Ras).

MATERIALS AND METHODS

Cell cultures and reovirus

The four canine MCT cell lines, VIMC (Takahashi et al., 2001), CoMS (Ishiguro et al., 2001), CM-MC (Takahashi et al., 2001) and HRMC (Ohmori et al., 2008), that were used in Chapter 1 were further exploited in the studies in this chapter. These cell lines were kindly provided by Dr. Takayuki Nakagawa and Dr. Hajime Tsujimoto. All cells were maintained in R10 complete medium (RPMI1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol) kept at 37 ˚C in a humidified 5% CO_2 incubator.

The Dearing strain of reovirus serotype 3 (Reolysin®; clinical grade reovirus; GMP) was obtained from Oncolytics Biotech Inc. (Calgary, Canada) through a collaborative effort. Stock virus was kept at -80 ˚C before being thawed and kept at 4 ˚C during usage.

Protein studies of reovirus-infected cell lines

Cells were either mock-infected or infected with reovirus at MOI 70 and harvested at specific time points depending on the susceptibility of each cell line to reovirus. Whole cell lysates that were used for Ras protein studies were treated with Mg-containing lysis buffer (1% NP40, 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 10% glycerol, 0.25% sodium deoxycholate) while NP40 lysis buffer (1% NP40, 10 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA) was used for the remaining protein studies. Both lysis buffers were supplemented with complete, Mini EDTA-free protease inhibitor mixture (Roche Diagnostics K.K., Tokyo, Japan). The Ras activation status of the reovirus-infected cells was evaluated after GST pull-down as previously described in Chapter 1. SDS-PAGE was performed to separate the proteins of interest, followed by protein transfer onto polyvinylidene fluoride (PVDF) membranes and probing with antibodies (Table III-1). The

membranes were visualized by immersion in Western Lightning Chemiluminescence reagent (PerkinElmer, CT, USA). Immunoreactive bands were visualized using the Luminescent Image Analyzer LAS 3000 mini (FUJIFILM, Tokyo, Japan) and analyzed using Science Lab 2005 (FUJIFILM). Membranes were stripped between antibody staining procedures with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl (pH 6.7)) for 30 minutes at 60 ˚C. Actin was used as loading controls in all the Western blots. All protein studies were carried out at least twice to confirm the findings. Band density of the Ras protein was measured using ImageJ before being analyzed with Microsoft Excel.

Total RNA extraction, cDNA synthesis & real-time PCR

RNA was harvested by extraction with TRI Reagent (Molecular Research Center, OH, USA) according to the manufacturer's protocol. RNA concentrations of 0.5 μg in a total volume of 6 μl were used in the reverse transcriptase reaction performed by using ReverTra Ace qPCR RT Master Mix with gDNA Remover according to the recommended protocol by the manufacturer (Toyobo, Osaka, Japan). cDNA was subjected to real-time PCR amplification using the QuantiTect SYBR Green PCR kit (QIAGEN, CA, USA) as previously described (Umeki et al., 2013). The primers used for assaying the canine *HRas*, *NRas*, *KRas4a*, *KRas4b* and *RPL32* were as follows: forward, 5'-CGAGAGATTCGACAGCACAA-3', and reverse, 5'-CCTCCTCCTTCCTCCTTCC-3'; forward, 5'-GCAGTGATGATGGGACTCAA-3', and reverse, 5'-CCTGTCCTACCTTGACAGTGG-3'; forward, 5'-AGACTCCTGGCTGTGTGAAAA-3', and reverse, 5'-TTCAAATCAGAATCCCACACC-3'; forward, 5'-TTCTGTCTTGGGGTTTTTGG-3', and reverse, 5'-GGGATGATTCGAAAGCTTCA-3'; forward, 5'-TGGTTACAGGAGCAACAAGAAA-3', and reverse, 5'-GCACATCAGCAGCACTTCA-3', respectively. PCR and fluorescence intensity detection

were performed with the CFX96 system (Bio-Rad Laboratories, CA, USA).

RESULTS

Reovirus downregulates and degrades Ras protein in canine MCT cell lines

Even though Ras transformation has been proven to promote reovirus disassembly, infectivity and more efficient virus release (Marcato et al., 2007), it is still unclear of the effects of reovirus on this pathway. We have reported in Chapter 1 that reovirus induces cell death in canine MCT cell lines without any correlation with the Ras activation status. Therefore, we investigated the effects of reovirus infection on the Ras signaling pathway, starting with the effect of reovirus on Ras activation. Results from the GST pull-down assay indicated that the level of GTP-bound Ras was reduced after reovirus infection in all four MCT cell lines (Fig. III-1A).

At the same time, the reduction of total Ras was detected as early as 12 hpi in the two cell lines that were the most susceptible to reovirus, VIMC and CoMS (Fig. III-1B). The level of total Ras in the other two cell lines remained unchanged even though more than 50% cell death (CM-MC at 24 hpi and HRMC at 72 hpi) was observed.

In order to elucidate if the reduction of Ras was due to disruption at mRNA synthesis or increased rate of Ras protein degradation, comparison of the mRNA levels of *Hras*, *Nras*, *Kras4a* and *Kras4b* was made between mock and reovirus-infected MCT cell lines. At time points where there were minimal cell death (12 hpi in VIMC, CoMS, CM-MC and 24 hpi in HRMC), none of the cell lines showed reduction of the *ras* mRNA levels (Fig. III-1C). This highly suggests that reovirus increases the rate of Ras degradation instead of disrupting the transcription of *ras* mRNA.

Reovirus downregulates the activation of Ras downstream proteins and Akt

Aligned with the discovery of the effects of reovirus on the Ras protein, investigation of the effects of reovirus on the Ras/Raf/MEK/ERK pathway was carried out. Examination of the Ras downstream proteins revealed that phosphorylation of Raf, MEK and ERK were reduced at 12 hpi for VIMC, CoMS and CM-MC while the reduction of the phosphorylation of these proteins in HRMC started to take place at 24 hpi (Fig. III-2A). This finding seems to agree with the level of susceptibility of the MCT cell lines towards reovirus.

Next, investigation was carried out on the PI3K/Akt pathway that interacts differently but closely with the Ras/Raf/MEK/ERK pathway (Steelman et al., 2011). The reduction of phosphorylated Akt occurred in VIMC, CoMS and CM-MC after reovirus infection but not in HRMC (Fig. III-2B). This discovery indicates that the inability of reovirus to downregulate the PI3K/Akt pathway is one of the key factors that reduces the susceptibility of the cells towards reovirus.

Apoptosis in canine MCT cell lines is dictated by Bim and the downregulation of phosphorylated Bad and Mcl-1

Proteins of the Bcl-2 family play a central role in apoptosis and both ERK and Akt (Steelman et al., 2011; Yancey et al., 2013) can control the regulation of these proteins. Since it is unsure if the downregulation of the Ras/Raf/MEK/ERK and/or PI3K/Akt pathway by reovirus is related to the induction of apoptosis, we investigated the roles of the pro-apoptotic (Bim and Bad) and anti-apoptotic (Mcl-1) of the Bcl-2 family in reovirus-infected MCT cell lines (Fig. III-3).

There are three known major isoforms of the pro-apoptotic Bim, which are the Bim EL, Bim L and Bim S (Miao et al., 2007). The expression of Bim, especially Bim EL, either increased or did not change after reovirus infection. Even though all three major isoforms of

Bim can be detected in the cell lines, the pattern of expression was different among the cell lines and we are unsure if this has any relationship with the ability of reovirus to induce apoptosis in these cell lines.

Phosphorylation of Bad promotes cell survival while Bad induces cell death (Tan et al., 2000). As expected in cells undergoing apoptosis, the phosphorylation of Bad was reduced after reovirus infection but the expression of Bad did not. Consistent with our hypothesis, the expression of the anti-apoptotic Mcl-1 was also reduced in all the reovirus-infected cells.

Reovirus downregulates the phosphorylation of c-Kit and c-Kit related Ras upstream proteins

Since c-Kit is one of the upstream effectors of the Ras signaling pathway (Linnekin, 1999), we examined the effects of reovirus on c-Kit and other c-Kit related Ras upstream effectors. From our results, not only was the phosphorylation of c-Kit reduced, c-Kit receptors were also degraded after reovirus infection. Reduction of the p-c-Kit and c-Kit expression took place as early as 12 hpi in all the cell lines (Fig III-4A). Even though both p-c-Kit and c-Kit expression was reduced drastically in HRMC after 12 hpi, cell viability of reovirus–infected HRMC still remained around 50% at 48 hpi (Chapter 1 Fig. I-2A). This suggests that there must be other pathway(s) that supports cell survival and prevent apoptosis in HRMC.

Influenced by the degradation of c-Kit, reduction of the phophorylation of PLCγ1, PLCγ2 and SHP-2 took place in VIMC and CoMS (Fig III-4B). However, downregulation of PLCγ2 did not take place in CM-MC. HRMC did not express any PLCγ2 and there was no phosphorylation of PLCγ1 and SHP-2 in HRMC, which further proves that other pathways are involved in the lower susceptibility of HRMC to reovirus.

DISCUSSION

So far, reports have only been focused on the relationship of EGFR and reovirus, but not on any other receptor tyrosine kinase upstream of Ras, such as c-Kit. Unlike EGFR, c-Kit is a member of the type III receptor tyrosine kinase family that is expressed by many cell types, especially in mast cells (Witte, 1990). Commonly associated with cell survival, proliferation, differentiation and apoptosis (Webster et al., 2006), c-Kit and its downstream signal transduction pathways in mast cells has been studied and described by many. Proven to be very complex, the known signal transduction pathways of c-Kit include the Ras/Raf/MEK/ERK, PI3K/Akt, PLC-γ, Src kinase and JAK/STAT pathway with some them working closely with each other (Liang et al., 2013; Roskoski, 2005).

In our study, reovirus dysregulated the expression of c-Kit, the Ras/Raf/MEK/ERK pathway and the PI3K/Akt pathway to induce apoptosis in the canine MCT cell lines. However, we were unable to tell if the dysregulation took place one after another or all at the same time. Based on the currently understanding of the signal transduction pathways, we deduce that reovirus can induce a dysregulatory cascade starting from the deactivation and degradation of c-Kit in the reovirus-infected cells. This will lead to the subsequent dysregulation of the Ras signaling pathway. At the same time, the cascade effect also influences the downregulation of PLC-γ and Akt in the cells.

On the other hand, reovirus can begin the disruption of normal cellular process by first dysregulating the Ras signaling pathway. Grb2 plays an important role in tumorigenesis (Dankort, 2001) and Grb2 forms complex with the guanylnucleotide exchange factor, Sos, in regulating Ras activity (Waters et al., 1995). Even though Sos has been previously reported to enhance reovirus cytotoxicity (Shmulevitz et al., 2005), the exact kinetics behind this is still unknown. Therefore, we hypothesize that the Grb2-Sos complex is disrupted after the dysregulation of the Ras signaling pathway. The disrupted Grb2-Sos complex, in turn,

induces the degradation of c-Kit via the recruitment of a ubiquitin ligase called cbl (Sun et al., 2007). At the same time, the reduction in c-Kit and Ras activity also downregulates the PI3K/Akt pathway. However, this theory remains to be proven with further studies.

The inability of reovirus to downregulate Akt in HRMC was an exciting discovery. The upstream signal of Akt comes from PI3K that can be affected by the tumor suppressor, PTEN. The mutations in PTEN have been reported in various human and canine cancers (Levine et al., 2002; Li et al., 1997; Steck et al., 1997). There is a possibility that PTEN is mutated in HRMC, which can lead to the accumulation of phosphinositides at the D3 position and hence, the constant activation of Akt (Aoki et al., 1998). In order to prove this theory, we plan to investigate the PTEN mutations in HRMC and the other cell lines in the future.

Akt has the ability to promote cell survival through the inhibition of *C. elegans* death gene *ced-3* possesses homology to interleukin-1β (IL-1β)-converting enzyme (CED-3/ICE)-like activity but does not alter Bcl-2 or Bcl-XL (Kennedy et al., 1997). As ERK can induce apoptosis through the effects of apoptotic proteins belonging to the Bcl-2 family (Cagnol and Chambard, 2010.), HRMC still undergoes apoptosis due to the dysregulated Ras/Raf/MEK/ERK pathway. This has been proven by the consistent pattern of Bim, phosphorylated Bad and Mcl-1 in all the cell lines (Fig. III-3). This finding also agrees that Akt does not interfere with the activities of Bcl-2 proteins. As the apoptosis induced by reovirus in HRMC solely relies on the dysregulated Ras/Raf/MEK/ERK pathway, susceptibility of HRMC to reovirus is notably lower than the other MCT cell lines. Therefore, it seems like the ability of reovirus to dysregulate the Ras signaling pathway and the concurrent downregulation of the PI3K/Akt pathway enhances the magnitude of the apoptotic event. However, further studies need to be carried out with transformation of Akt-related genes in reovirus-susceptible cells to confirm the inhibition of cell death.

Even though there are a lot of reports on the Ras signaling pathway contributing to

reovirus replication and spread, information on the effects of reovirus in cell signaling pathways was scarce. One recent report focused on the effects of reovirus in TRAIL-resistant gastric cancer cells. Similar to our study, reovirus was not only able to downregulate the activation of Ras and ERK, but also reduced the phosphorylation of Akt in the process of inducing apoptosis in the TRAIL-resistant cells (Cho et al., 2010). Another unrelated report have also shown that reovirus induces apoptosis via the dephosphorylation of ERK and at the same time, increases the activation of the JNK-dependent transcription factor, c-Jun (Clarke et al., 2001).

Besides reovirus, it has been reported that other naturally occurring or attenuated viruses, such as vesicular stomatitis virus, mumps, Newcastle disease virus and measles virus, have oncolytic potential, which is dependent on the Ras and Ras-downstream PKR activity (Davis and Fang, 2005). Since approximately 30-40% of cancers harbors Ras activating mutations (Bos, 1989), herpes simplex virus I, Influenza A and adenovirus 5 have been genetically altered specifically as oncolytic viruses that targets tumor cells with activated Ras and are PKR-deficient (Davis and Fang, 2005). Therefore, it seems like the involvement of Ras and its downstream protein, PKR, is not just exclusive to reovirus.

In our study, low phosphorylation level of Raf, MEK, ERK, Akt and Bad was observed at 0-time point in both the mock and reovirus-infected VIMC, CoMS and CM-MC. This was most probably due to the 4 ˚C incubation for one hour before cell collection that is necessary to allow adequate viral attachment. Low temperatures can reduce the activation of signaling pathways and cause cells to be in a state of quiescence, which is reversible upon the increase of temperature (Chan et al., 1999). However, we are unsure why this incubation step did not affect HRMC.

Our findings also showed the lack of involvement of PLCγ2 in CM-MC and PLCγ1, PLCγ2 and SHP-2 in HRMC (Fig. III-4B). It seems like a coincidence that the pattern of

reoviral susceptibility depends on the involvement of these proteins, where they were downregulated in cells highly susceptible to reovirus (VIMC and CoMS) but partially involved in CM-MC and not involved in HRMC. As these proteins are upstream proteins of Ras (Roskoski, 2005), these proteins might be crucial in the regulation of cell survival and proliferation in VIMC and CoMS. Therefore, the ability of reovirus to disrupt these proteins can lead to dysregulation in signaling cascades. However, we are unsure of the exact proteins and mechanisms that might be involved.

The complexity of cellular events that occurs after virus infection only proves that all viruses are unique and have various tactics to outsmart and survive in the host cells. Differences in intracellular signaling pathways among cell types has allowed some cells to be susceptible to virus infection while enhancing viral resistance in other cells. Even though Ras transformation has been proven to promote reovirus disassembly, infectivity and more efficient viral progeny release (Marcato et al., 2007), the key determinant molecule of reoviral susceptibility is still unknown.

From our studies, reovirus dysregulates the Ras/Raf/MEK/ERK pathway and degrades c-Kit as a common mean to induce apoptotic cell death in all the canine MCT cell lines. But the level of reoviral susceptibility differs at the ability of reovirus to downregulate the PI3K/Akt pathway. At this point, we only managed to focus on how reovirus utilizes two of the major cellular pathways to induce apoptosis. It only sounds logical that reovirus also exploits other proteins and pathways that are yet to be discovered. As cell signaling pathways often interact with each other, complication arises to pinpoint the exact mechanism that dictates the actions of reovirus. Perhaps, this involves something that might be beyond our current understanding. Therefore, further studies are definitely needed to provide concrete evidence to depict the bigger picture of reovirus oncolysis.

TABLE

Table III-1: Antibodies used for Western blotting

No.	Antibody	Product no.	Source
Primary antibodies			
1.	Mouse monoclonal anti-pan-Ras	#OP40	Calbiochem, MA, USA
2.	Rabbit monoclonal anti-phospho-c-Raf	#9427	Cell Signaling, MA, USA
3.	Mouse anti-c-Raf	R19120	BD Biosciences, Ontario, Canada
4.	Rabbit monoclonal anti-phospho-MEK1/2	#9154	Cell Signaling
5.	Rabbit polyclonal anti-MEK-1	sc-219	Santa Cruz Biotechnology, CA, USA
6.	Rabbit monoclonal anti-phospho-p44/42 MAPK (ERK1/2)	#4370	Cell Signaling
7.	Rabbit polyclonal anti-ERK 2	sc-154	Santa Cruz Biotechnology
8.	Rabbit monoclonal phospho-Akt	#4060	Cell Signaling
9.	Rabbit monoclonal anti-Akt	#4691	Cell Signaling
10.	Rabbit polyclonal anti-Mcl-1	#613601	BioLegend, CA, USA
11.	Rabbit monoclonal anti-Bim	#2933	Cell Signaling
12.	Rabbit monoclonal anti-phospho-Bad	#5284	Cell Signaling
13.	Rabbit monoclonal anti-Bad	#9239	Cell Signaling
14.	Rabbit polyclonal anti-phospho-c-Kit	#3391	Cell Signaling
15.	Goat polyclonal anti-c-Kit	sc-1494	Santa Cruz Biotechnology
16.	Rabbit polyclonal anti-phospho-PLCγ1	#2821	Cell Signaling
17.	Rabbit monoclonal anti-PLCγ1	#5690	Cell Signaling
18.	Rabbit polyclonal anti-phospho-PLCγ2	#3871	Cell Signaling
19.	Rabbit polyclonal anti-PLCγ2	#3872	Cell Signaling
20.	Rabbit polyclonal anti-phospho-SHP-2	#3751	Cell Signaling
21.	Rabbit monoclonal anti-SHP-2	#3397	Cell Signaling
22.	Goat polyclonal anti-actin	sc-1615	Santa Cruz Biotechnology
23.	Mouse monoclonal anti-actin	A5441	Sigma-Aldrich, MO, USA

Secondary antibodies			
1.	Goat anti-rabbit IgG HRP	62-6120	Zymed Laboratories, CA, USA
2.	Goat anti-mouse IgG HRP	62-6520	Zymed Laboratories
3.	Rabbit anti-goat IgG HRP	A50-100P	Bethyl Laboratories, TX, USA

Figure 1

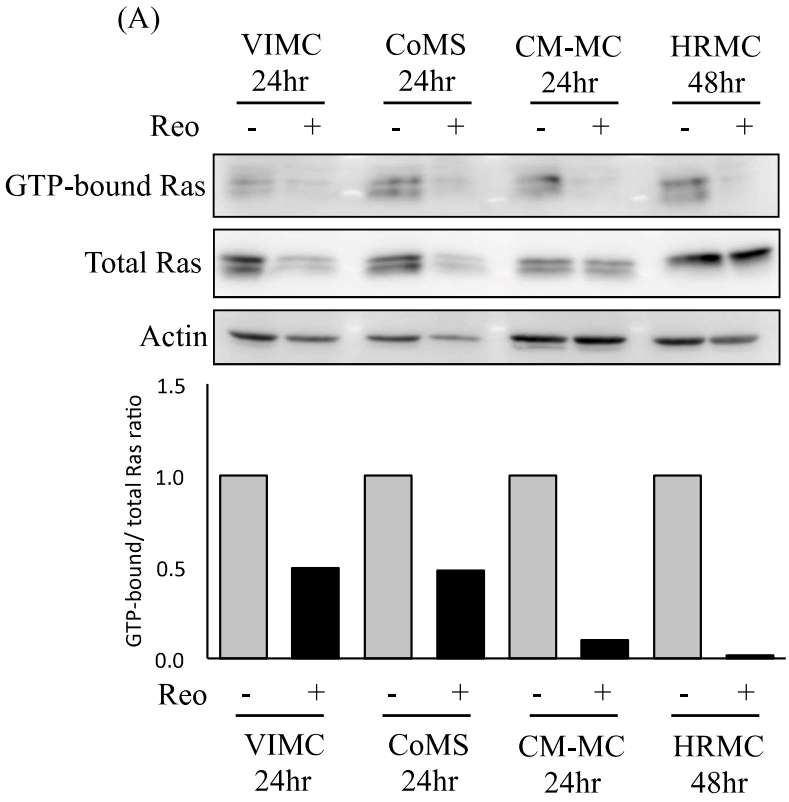

(A)
VIMC 24hr
CoMS 24hr
CM-MC 24hr
HRMC 48hr
Reo - + - + - + - +
GTP-bound Ras
Total Ras
Actin
GTP-bound/ total Ras ratio
1.5
1.0
0.5
0.0
Reo - + - + - + - +
VIMC 24hr
CoMS 24hr
CM-MC 24hr
HRMC 48hr

Figure 1

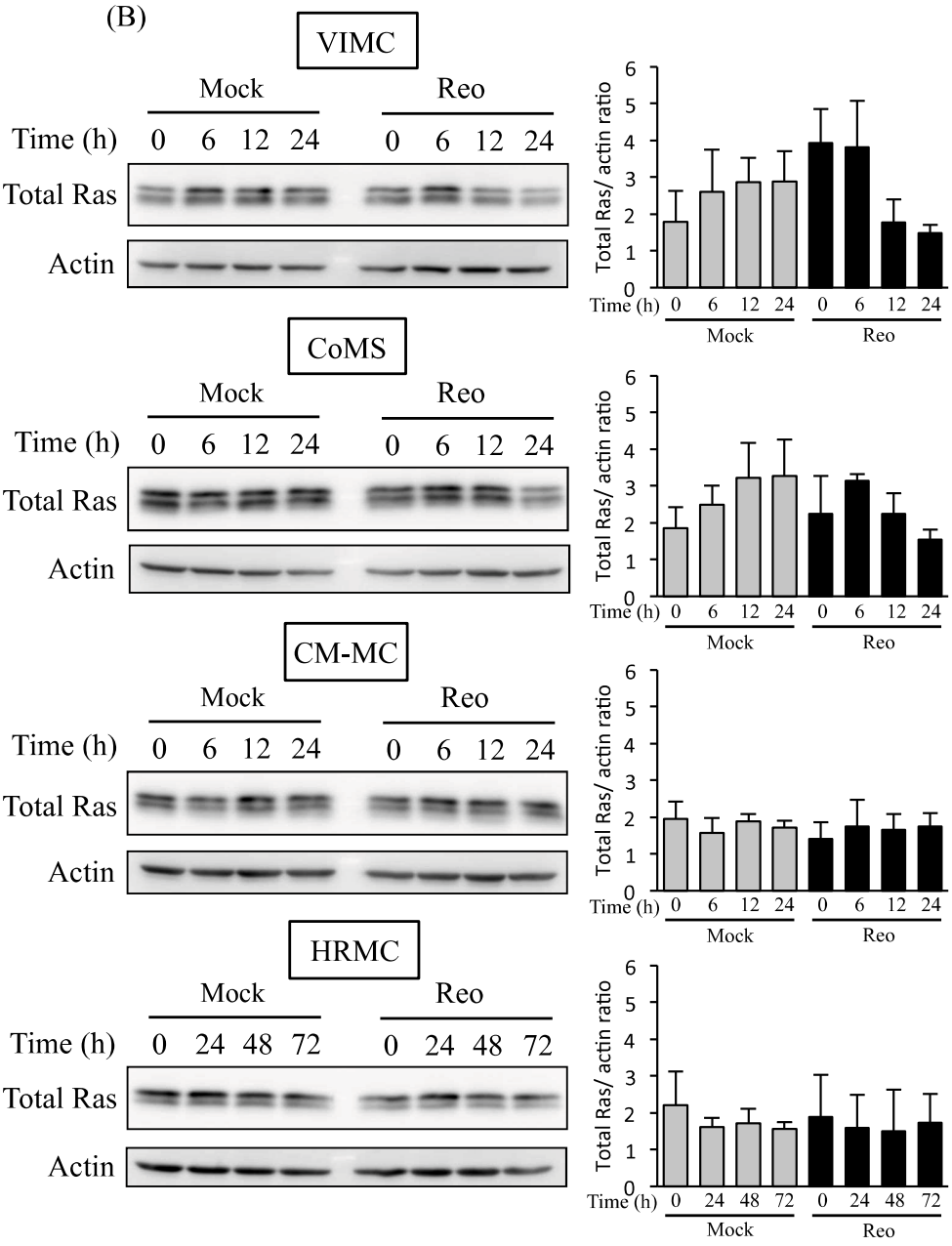

(B)
VIMC
Mock
Reo
Time (h) 0 6 12 24 0 6 12 24
Total Ras
Actin
Total Ras/ actin ratio
CoMS
CM-MC
HRMC
Time (h) 0 24 48 72 0 24 48 72

Figure 1

(C)

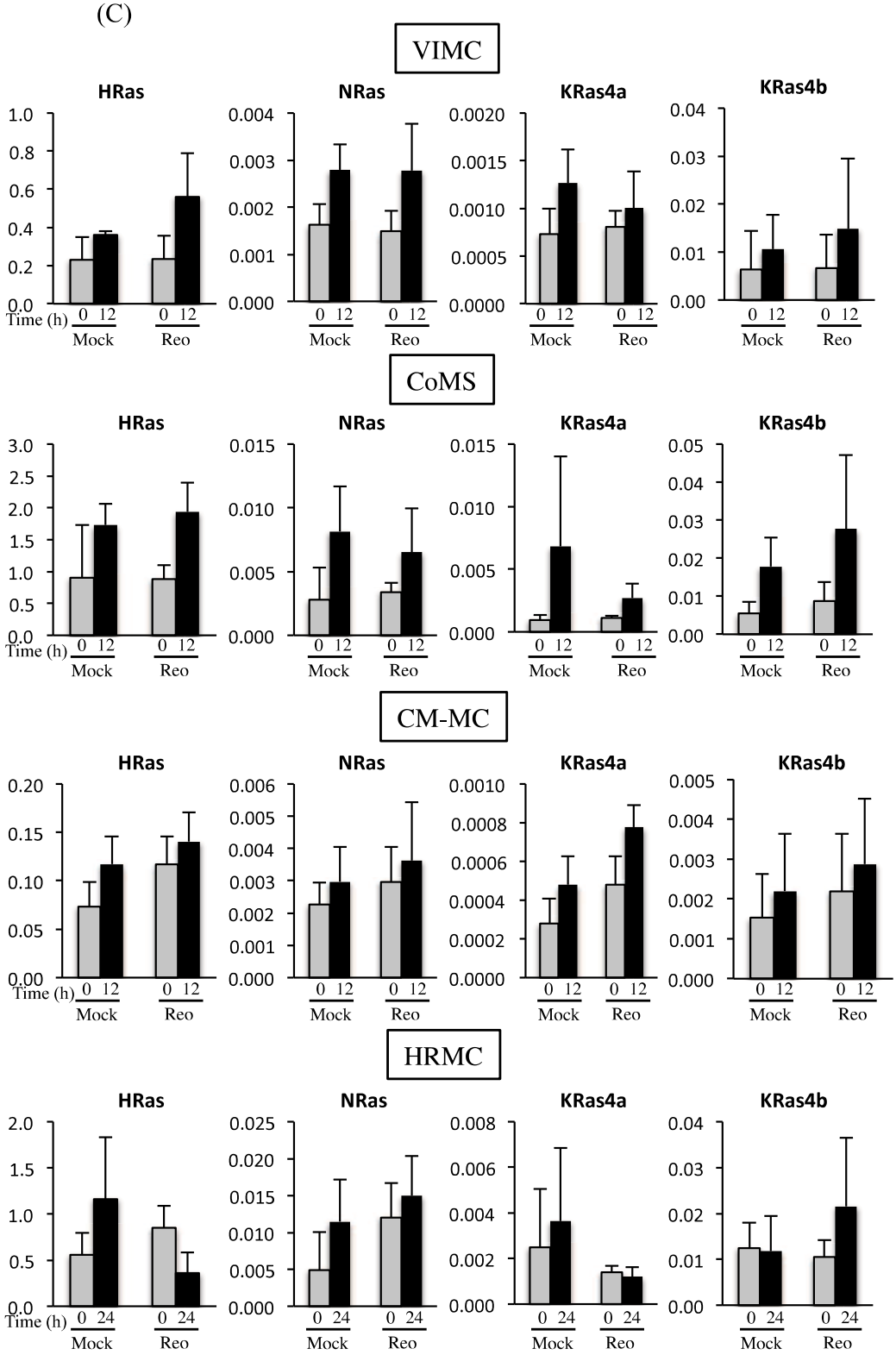

VIMC
HRas
NRas
KRas4a
KRas4b
Time (h) 0 12 0 12
Mock Reo
CoMS
HRas
NRas
KRas4a
KRas4b
Time (h) 0 12 0 12
Mock Reo
CM-MC
HRas
NRas
KRas4a
KRas4b
Time (h) 0 12 0 12
Mock Reo
HRMC
HRas
NRas
KRas4a
KRas4b
Time (h) 0 24 0 24
Mock Reo

Fig. III-1. Reovirus downregulates and degrades Ras protein in canine MCT cell lines. (A) Cells were infected with reovirus (MOI 70) before being collected at 24 hpi (48 hpi for HRMC). GST-RBD protein was extracted and immobilized on glutathione-Sepharose beads to affinity-precipitated Ras-GTP from cell lysates. The affinity-precipitated Ras-GTP and whole cell lysates were subjected to SDS-PAGE before detection by Western blotting with anti-Ras Ab. Protein band density was measured using ImageJ. The ratio of GTP-bound/ total Ras is shown in the bar graph with mock-infected samples calculated as 1.0. (B) Whole cell lysates of reovirus-infected (MOI 70) canine MCT cell lines at 0, 6, 12 and 24 hpi (0, 24, 48 and 72 hpi for HRMC) were prepared before proteins were separated using SDS-PAGE. The level of total Ras was determined using anti-Ras Ab. β-actin was used as protein loading controls. Protein band density was measured using ImageJ. Mean ± SD, n = 3. (C) Reovirus-infected (MOI 70) canine MCT cell lines were collected at 0 and 12 hpi (24 hpi for HRMC) before total mRNA was extracted and cDNA synthesis. Real-time PCR was performed to detect the expression level of *Hras*, *Nras*, *Kras4a* and *Kras4b* mRNA. Gene expression was normalized to *RPL32*. Mean ± SD, n = 3.

Figure 2

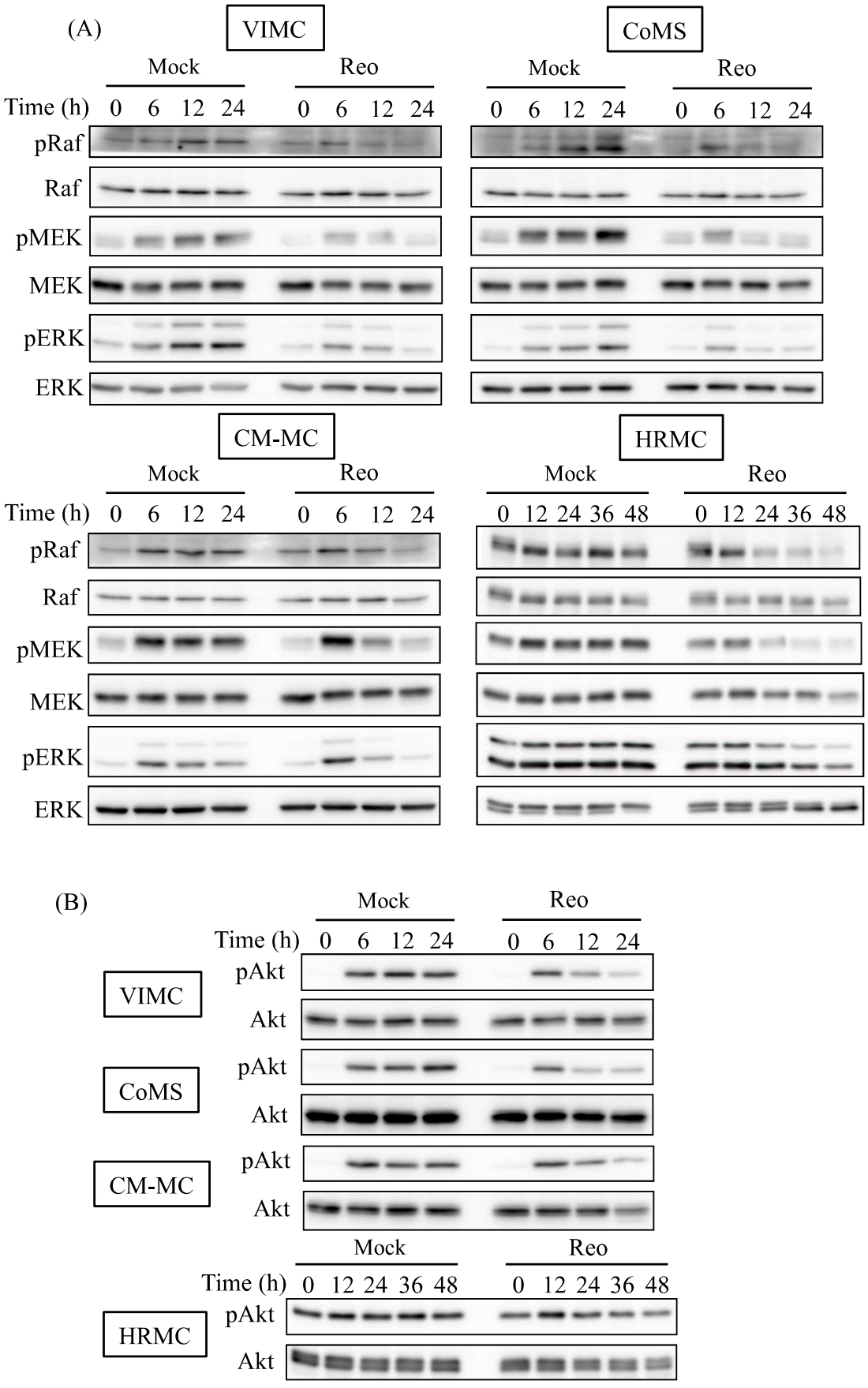

(A)
VIMC
CoMS
CM-MC
HRMC
Mock
Reo
Time (h)
0 6 12 24
0 12 24 36 48
pRaf
Raf
pMEK
MEK
pERK
ERK
(B)
pAkt
Akt
VIMC
CoMS
CM-MC
HRMC

Fig. III-2. Reovirus downregulates the activation of Ras downstream proteins and Akt. Whole cell lysates of reovirus-infected (MOI 70) canine MCT cell lines at 0, 6, 12 and 24 hpi (0, 24, 36 and 48 hpi for HRMC) were prepared before proteins were separated using SDS-PAGE. Phosphorylated and total Raf, MEK, ERK (A) and Akt (B) were detected with Western blotting using antibodies summarized in Table III-1.

Figure 3

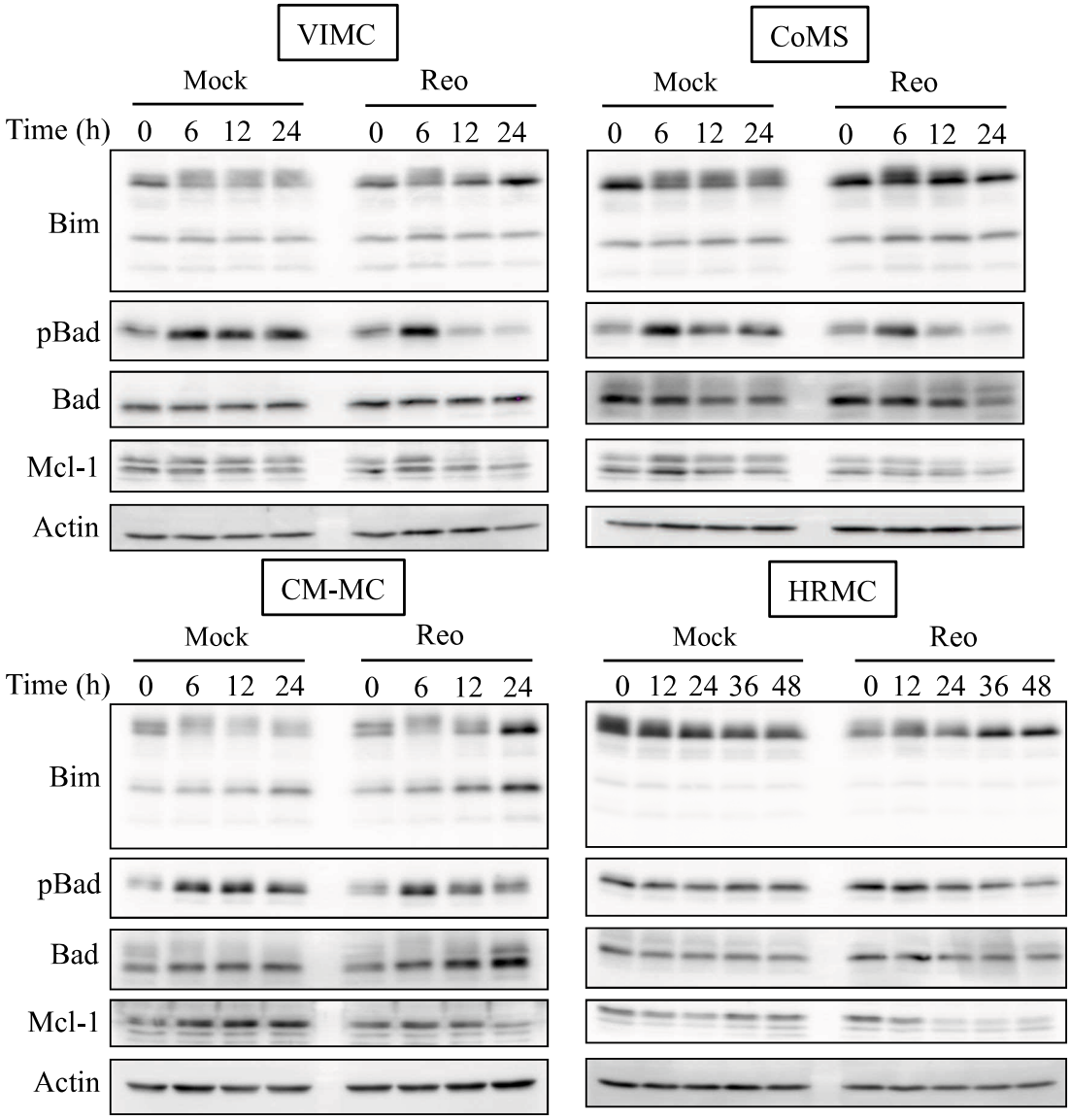

VIMC
Mock
Reo
Time (h)
0 6 12 24 0 6 12 24
Bim
pBad
Bad
Mcl-1
Actin
CoMS
Mock
Reo
0 6 12 24 0 6 12 24
CM-MC
Mock
Reo
Time (h)
0 6 12 24 0 6 12 24
Bim
pBad
Bad
Mcl-1
Actin
HRMC
Mock
Reo
0 12 24 36 48 0 12 24 36 48

Fig. III-3. Apoptosis in canine MCT cell lines is dictated by Bim and the downregulation of phosphorylated Bad and Mcl-1. Whole cell lysates of reovirus-infected (MOI 70) canine MCT cell lines at 0, 6, 12 and 24 hpi (0, 24, 36 and 48 hpi for HRMC) were prepared before proteins were separated using SDS-PAGE. Bim, phosphorylated Bad, total Bad and Mcl-1 were detected with Western blotting using antibodies summarized in Table III-1.

Figure 4

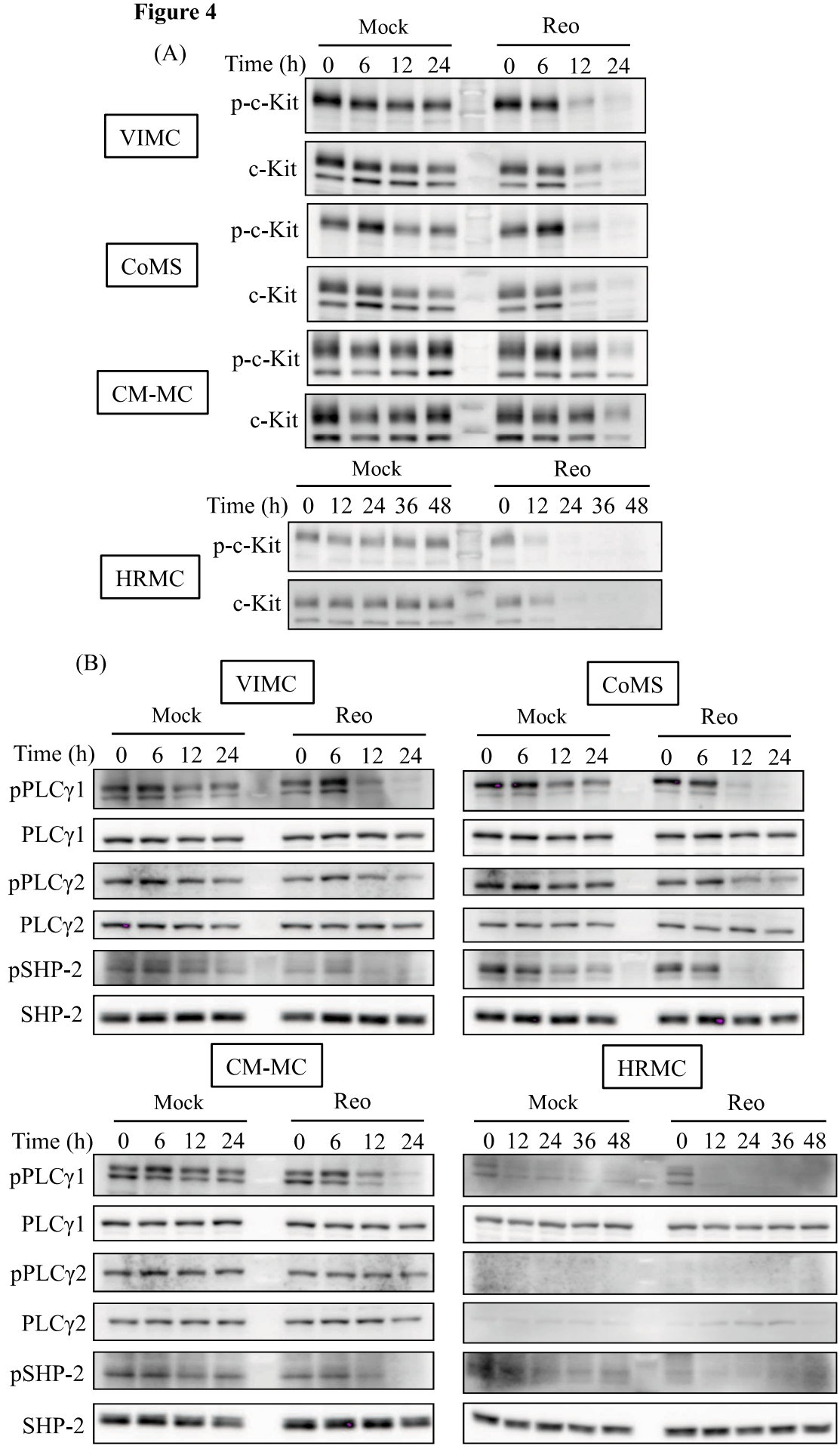

(A)
Mock
Reo
Time (h) 0 6 12 24 0 6 12 24
p-c-Kit
c-Kit
VIMC
CoMS
CM-MC
Time (h) 0 12 24 36 48 0 12 24 36 48
HRMC
(B)
pPLCγ1
PLCγ1
pPLCγ2
PLCγ2
pSHP-2
SHP-2

Fig. III-4. Reovirus downregulates the phosphorylation of c-Kit and c-Kit related Ras upstream proteins. Whole cell lysates of reovirus-infected (MOI 70) canine MCT cell lines at 0, 6, 12 and 24 hpi (0, 24, 36 and 48 hpi for HRMC) were prepared before proteins were separated using SDS-PAGE. Phosphorylated and total c-Kit (A), PLCγ1, PLCγ1 and SHP-2 (B) were detected with Western blotting using antibodies summarized in Table III-1.

Figure 5

Reovirus susceptibility: VIMC & CoMS > CM-MC > HRMC

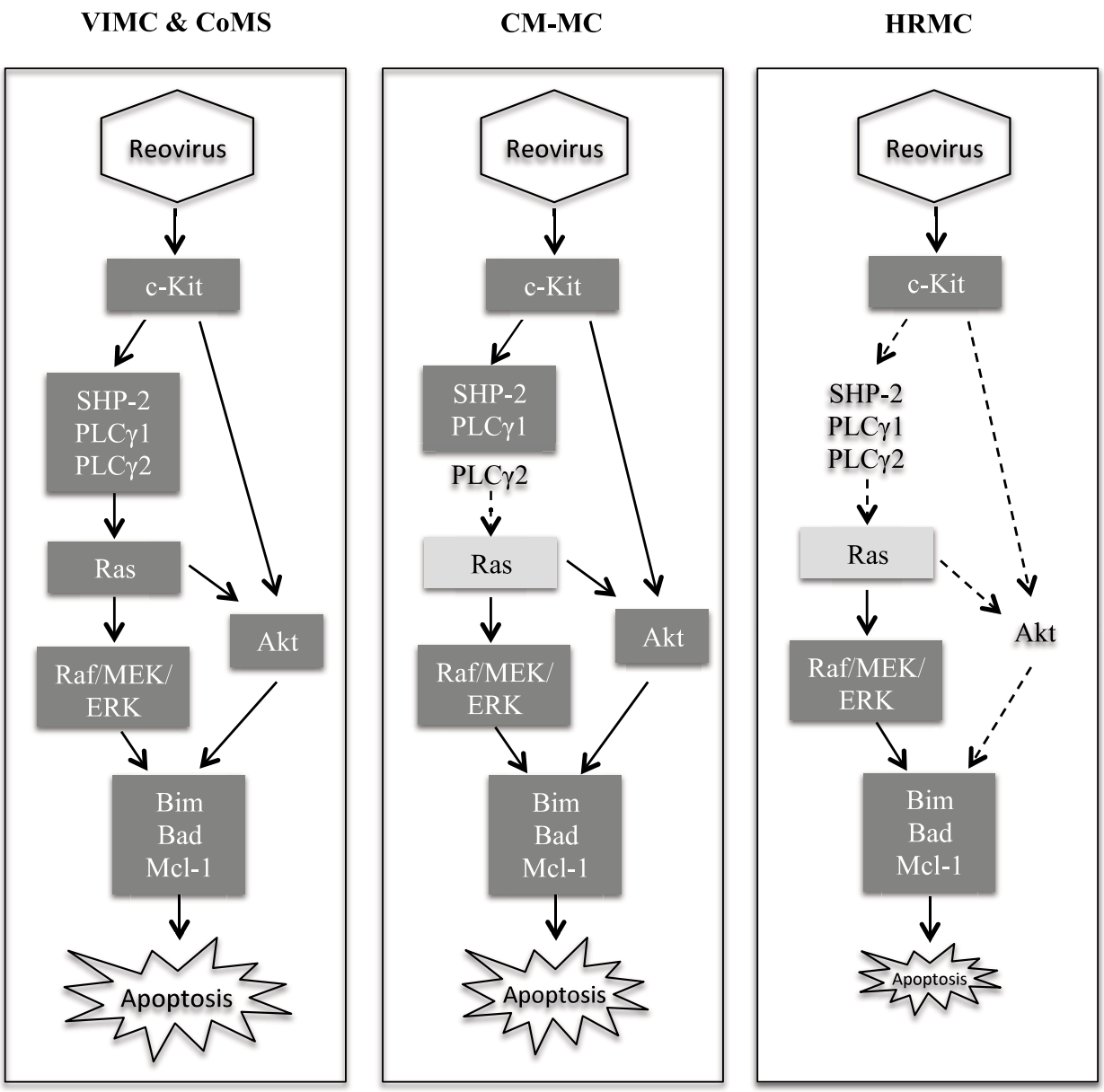


Fig. III-5. Diagrams depicting the effects of reovirus on various Ras downstream and upstream proteins that govern the susceptibility of canine MCT cell lines towards reovirus. Proteins highlighted in dark grey indicate the downregulation of protein activation/ expression after reovirus infection; proteins highlighted in light grey were partially affected by reovirus infection; proteins that are not highlighted were not affected by reovirus. Solid arrows represent direct relationship between proteins; dash arrows represent the lack of relationship between proteins. Size of shapes of apoptosis represent the magnitude of the apoptotic event.

CHAPTER 4

Seroepidemiology of reovirus in healthy dogs in six prefectures in Japan

SUMMARY

Reovirus infection is common in mammals. However, seroepidemiological data of reovirus neutralizing antibodies are limited in dogs. In this chapter, sera of 65 healthy dogs from six prefectures across Japan were tested for neutralizing antibodies against reovirus serotype 1 strain Lang (T1L), serotype 2 strain Amy (T2A) and serotype 3 strain Dearing (T3D) using plaque reduction neutralization test (PRNT). Seropositivity against reovirus T1L, T2A and T3D was 53.85%, 33.85% and 46.15%, respectively. Distribution of reovirus seropositive samples displayed no distinguishable geographical pattern. However, reovirus seropositivity increases with age and in dogs housed outdoor. Co-infection of multiple reovirus serotypes in dogs is also possible. These data will provide valuable insights towards the usage of reovirus in oncolytic virotherapy in canine cancers.

INTRODUCTION

Reovirus is ubiquitous in geographical distribution and has the capacity to infect nearly every known mammalian species, including humans and dogs (Rosen, 1962). However, as a single agent, reovirus rarely causes clinical disease. Upper respiratory or gastrointestinal symptoms are among the possible manifestations of reovirus infection in young and adult animals (Kokubu et al., 1993; Lou et al., 1963; Mochizuki and Uchizono, 1993). Reovirus has also been reported to be one of the aetiologies of kennel cough (Buonavoglia and Martella, 2007).

Seroepidemiological surveys of reovirus in healthy humans revealed that the incidence of seropositivity rises from approximately 35% in early childhood, to approximately 60% in teenage years and more than 85% in late adulthood (Lerner et al., 1962; Selb and Weber, 1994; Tai et al., 2005). However, unlike in humans, seroepidemiological data of reovirus in healthy dogs are limited. Reports have indicated that 14-63% of sampled dog populations have an elevated reovirus neutralizing antibody titer (Decaro et al., 2005; Fukumi et al., 1969; Murakami and Kato, 1975; Osterhaus et al., 1977). Even though isolation of various serotypes of reovirus from dogs and cats has been reported, it is usually incidental (Binn et al., 1977; Csiza, 1974; Kokubu et al., 1993; Lou et al., 1963; Marshall et al., 1987; Massie and Shaw, 1966; Mochizuki and Uchizono, 1993; Scott et al., 1970). Alternatively, reovirus infection can be detected, and reovirus serotypes are distinguishable by means of the capacity of reovirus neutralizing antibodies to neutralize viral infectivity and inhibit hemagglutination (HA) (Rosen, 1960; Sabin, 1959). Reovirus neutralization and HA activities are restricted to a single reovirus gene segment, S1, that encodes for the σ1 and σ1s proteins (Weiner and Fields, 1977).

It has been reported that the dramatic increment of reovirus neutralizing antibody titer hampers the efficiency of intravenous reovirus therapy in human cancer patients (White et al.,

2008). Therefore, reovirus neutralizing antibodies due to natural infection may also interfere with reovirus therapy. This emphasizes the importance of seroepidemiological data of reovirus in the dog population in order to allow a sound prediction of the effects of therapy using reovirus in canine cancer patients.

This chapter focused on the seroepidemiological survey of reovirus serotype 1 strain Lang (T1L), serotype 2 strain Amy (T2A) and serotype 3 strain Dearing (T3D) in healthy dogs from six prefectures across Japan, namely Hokkaido, Tokyo, Aichi, Osaka, Yamaguchi and Fukuoka. Reovirus seropositive samples were also analyzed according to age groups, housing environment and co-infectivity of reovirus serotypes.

MATERIALS AND METHODS

Mouse L929 fibroblastic cell line was used throughout the study. The cell line was obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) and maintained in R10 complete medium (RPMI1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol) kept at 37˚C in a humidified 5% CO_2 incubator. Reovirus T1L and T2A are stock viruses in our laboratory, while reovirus T3D was from Dr. Patrick W. K. Lee through Dr. Tsuyoshi Etoh (Oita University, Japan). All three serotypes of reovirus were propagated in L929 cells grown in RPMI1640 without FBS. Supernatant of the infected cells was collected when cytopathic effect was observed in 80% of the cells. Viral titer was determined by plating the viral supernatant onto susceptible L929 cell monolayer using plaque titration method as previously described (Berard and Coombs, 2009). Briefly, virus was diluted serially and 200 μl of each dilution added to wells in six-well plates. After absorption for one hour at 37˚C, the cells were overlaid with two ml of RPMI1640 containing 0.8% Seaplaque Agarose (Lonza, Rockland, ME, U.S.A.) and antibiotics without FBS. After six days of incubation at 37˚C in a humidified 5% CO_2 incubator, plaques were fixed with 10% formalin and stained with crystal violet before being counted.

Serum was collected from a total of 65 healthy dogs that came to veterinary clinics for routine health checks in six prefectures (Hokkaido, Tokyo, Aichi, Osaka, Yamaguchi and Fukuoka) in Japan in 2006. All sera were stored at -20˚C and inactivated at 56˚C for 30 minutes prior to plaque reduction neutralization test (PRNT). A minimum of 10 samples from each prefecture was used in this study. PRNT was performed using L929 cell monolayer as previously described (Weiner and Fields, 1977) with modifications. To screen for reovirus seropositive samples, sera were diluted at 1:20, and 60 PFUs of reovirus was mixed before incubation for one hour at 37˚C. Next, the mixtures were incubated with the L929 cell

monolayer for another hour at 37˚C, 5% CO_2. Finally, the mixtures were removed before RPMI1640 containing 0.8% Seaplaque Agarose and antibiotics without FBS was layered onto the cells and incubated for 6 days. Plaques were fixed with 10% formalin and stained with crystal violet before being counted. Sera that reduce greater than 80% of plaques were considered positive for reovirus neutralizing antibodies (Weiner and Fields, 1977). Sera that were positive for reovirus neutralizing antibodies were selected, and PRNT was repeated with dilutions of serum up to 1:10,240 to determine the maximum antibody titer.

RESULTS AND DISCUSSION

Frequency distributions of neutralizing antibody titers against reovirus T1L, T2A and T3D are shown in Table IV-1. Almost half of the samples do not have neutralizing antibodies against reovirus T1L, T2A and T3D. There is no obvious difference between the frequencies of reovirus seropositivity among the three serotypes of reovirus. This indicates that reovirus infection is widely spread in the sampled dog population regardless of serotype. Distributions of reovirus seropositive samples in each of the six prefectures across Japan were also analyzed. However, there is an uneven distribution of reovirus neutralizing antibody titers with no distinguishable pattern in those prefectures (Fig. IV-1). Although the number of samples is limited, this suggests that reovirus is prevalent in the six prefectures involved in this study.

Next, we investigated the correlation of reovirus seropositivity in various age groups and housing environment of the sampled canine population. There is a trend in reovirus seropositivity according to age groups (Fig. IV-2A). The number of reovirus seropositive samples in young dogs (less than two years) is comparatively lower than old dogs (more than 11 years). This finding is consistent with a report of reovirus seropositivity in humans (Selb and Weber, 1994), where the percentage of reovirus seropositivity rises due to increased exposure to reovirus in later parts of life. The housing environment of dogs also correlates with the number of reovirus seropositive samples (Fig. IV-2B). In comparison to dogs housed indoor, dogs housed outdoor or in both environments have a higher percentage of reovirus seropositivity. As reovirus is commonly isolated from contaminated water sources (Matsuura et al., 1984; Muscillo et al., 2001; Tani et al., 1995), dogs housed outdoor are prone to exposure to reovirus. This result coincides with a report where a higher percentage of stray dogs were found to be reovirus seropositive (Fukumi et al., 1969).

In order to detect the co-infection of reovirus serotypes in the sampled population, we

created three comparison groups (T1L vs T2A; T1L vs T3D; T2A vs T3D) (Fig. IV-3). More than a quarter of the samples in all groups were seropositive for two reovirus serotypes. Out of the 65 sera, 15 were also seropositive for all three reovirus serotypes. This finding indicates that reovirus neutralizing antibodies of one serotype do not provide immunity towards reovirus infection of another serotype. Hence, reovirus infection of more than one serotype can still occur depending on exposure. Similar trends of reovirus infection of multiple serotypes have been previously reported in healthy dogs (Fukumi et al., 1969; Murakami and Kato, 1975; Osterhaus et al., 1977), cattle (Rosen et al., 1963), swine (Hirahara et al., 1988) and humans (Ouattara et al., 2011; Pal and Agarwal, 1968; Taylor-Robinson, 1965). This finding has provided important information suggesting that neutralizing antibodies against reovirus T1L and T2A will not affect the usage of reovirus T3D as therapy in canine cancers.

In summary, there is no difference in the seroprevalence of the three reovirus serotypes in healthy dogs according to geographical distribution. However, exposure to reovirus infection increases with age and in dogs housed outdoor. Co-infection of various reovirus serotypes is not uncommon in dogs and depends on the exposure to the virus. Reovirus seropositivity in the healthy dog population is high and comparable to that of the human population. This chapter has provided a better understanding of the seroepidemiology of reovirus in dogs. On top of that, these data are essential in predicting the outcome of oncolytic virotherapy using reovirus in canine cancers.

TABLE

Table IV-1. Summary of reovirus neutralizing antibody titers in healthy dogs in 6 prefectures in Japan.

Serotypes of reovirus		Neutralizing antibody titer								Total
		<20	20	40	80	160	320	640	1280	
T1L	No. of samples	30	5	9	8	6	2	3	2	65
	%	46.15	7.69	13.85	12.31	9.23	3.08	4.62	3.08	100.00
T2A	No. of samples	43	5	3	4	3	7	0	0	65
	%	66.15	7.69	4.62	6.15	4.62	10.77	0.00	0.00	100.00
T3D	No. of samples	35	9	5	6	3	2	5	0	65
	%	53.85	13.85	7.69	9.23	4.62	3.08	7.69	0.00	100.00

a) Samples are considered as reovirus seropositive when reovirus neutralizing antibody titer is 20 or greater.

FIGURES

Figure 1

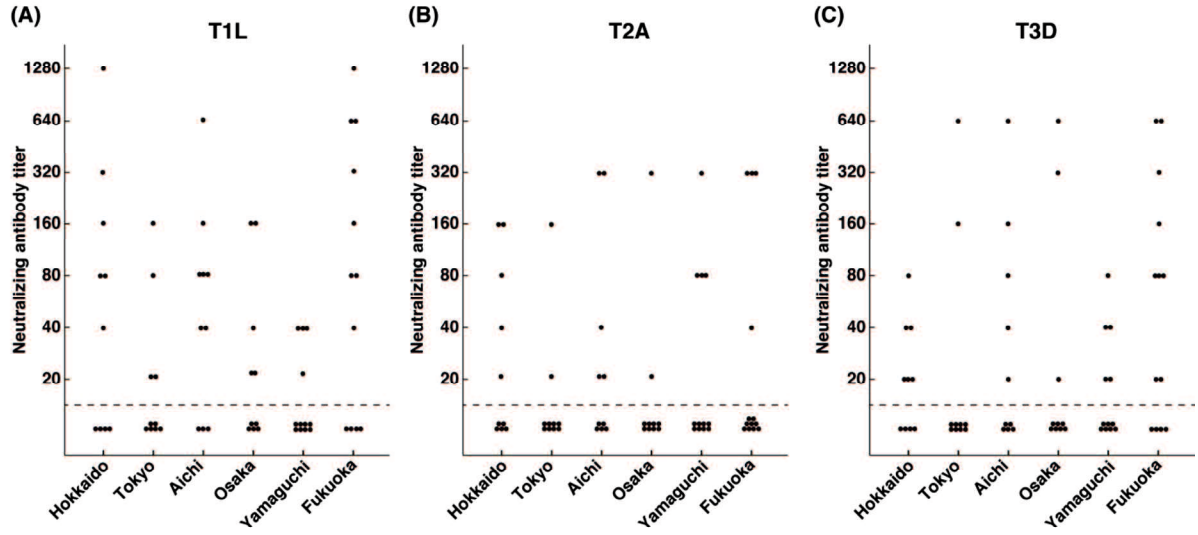


Fig. IV-1. Distributions of neutralizing antibody titers against reovirus T1L (A), T2A (B) and T3D (C) in Hokkaido, Tokyo, Aichi, Osaka, Yamaguchi and Fukuoka. Samples are considered as reovirus seropositive when reovirus neutralizing antibody titer is 20 or greater.

Figure 2

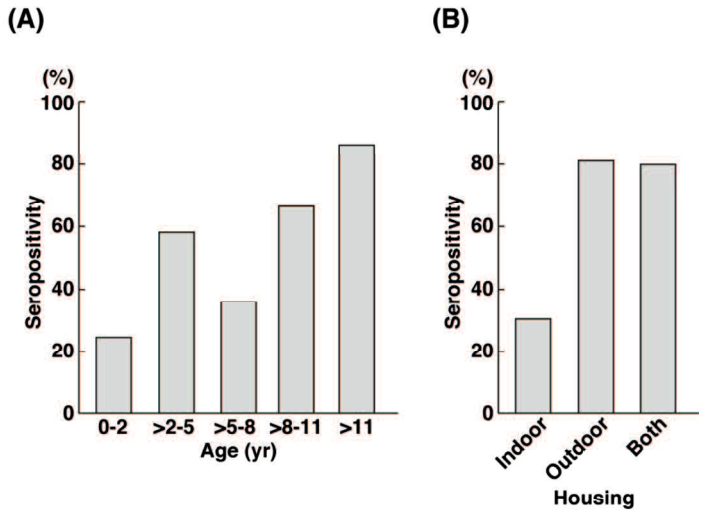


Fig. IV-2. Percentage of reovirus seropositivity according to age groups (A) and housing environment (B). Samples are considered as reovirus seropositive, if they are seropositive for any one of the reovirus serotypes. Reovirus seropositive samples have reovirus neutralizing antibody titer of 20 or greater.

Figure 3

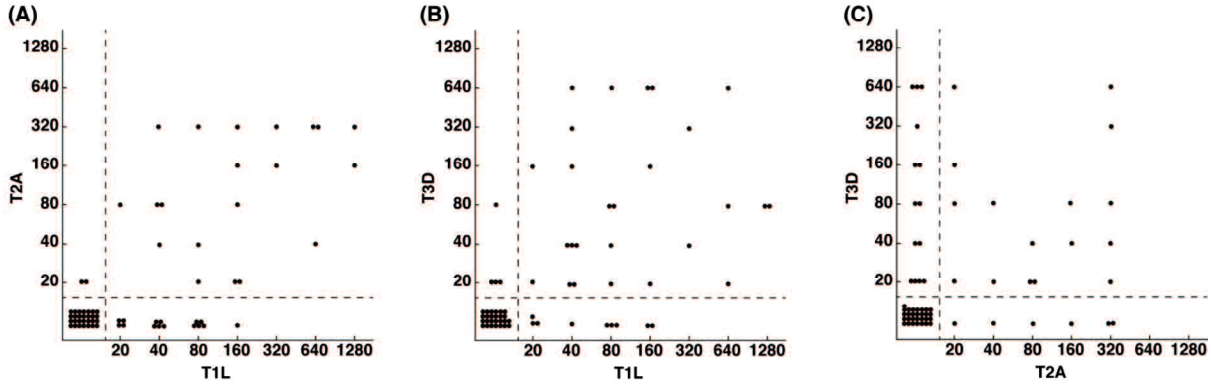


Fig. IV-3. Co-infection of reovirus T1L and T2A (A), T1L and T3D (B) and T2A and T3D (C). Samples are considered as reovirus seropositive when reovirus neutralizing antibody titer is 20 or greater.

CHAPTER 5

Experimental infection of reovirus in healthy dogs and preliminary studies of reovirus administration in canine cancer patients

SUMMARY

Due to the limited information on experimental infection of reovirus (serotype 3 Dearing strain) in dogs, we administrated three consecutive subcutaneous injections of reovirus at 1.4 x 10^8 $TCID_{50}$ in healthy dogs. No toxicities and viral shedding was provoked but a healthy anti-viral immune response was stimulated. In order to translate our laboratory results into clinical data, a maximum of 5 x 10^9 $TCID_{50}$ of reovirus has been administered either intratumorally or intravenously to canine patients bearing various types of cancer, which was found to be safe and well-tolerated. The adverse events after the administration of reovirus seen in the patients were mild and did not require any medical intervention. Viral shedding was infrequent and short-lived, denying any possibility of posing harm to their caretaker or other dogs. The anti-viral response that was detected in all canine patients has indicated that the concomitant usage of reovirus with an immunosuppressing agent might be favorable. Even though the determination of the efficacy of reovirus was not our objective, assessment of tumor size has been challenging in most of the patients. Therefore, for future studies, the evaluation of histological samples and the detection of reovirus protein in biopsied samples are needed to monitor the anti-tumoral effects of reovirus.

INTRODUCTION

Clinical trials are research studies involving actual patients suffering from certain diseases with the objective of testing new strategies in prevention, detection, diagnosis or treatment of those diseases. Since actual patients are involved, clinical trials are divided into various phases and conducted in a way that minimizes any possible harm to the patients. Generally, clinical trials are divided into four phases. Phase I studies mainly involve a minimum of 20 patients, whom which standard treatments are ineffective. The aim of phase I studies are to evaluate the safety of a new agent by determining the maximum tolerated dose (MTD) and its possible side effects. The phase II studies will ensue if the new agent does not induce any unacceptable toxicities assessed in the phase I studies. Phase II studies aim to obtain preliminary data on the efficacy of the new agent with a specific type of disease in a group of less than 300 patients.

While phase III studies compare the effectiveness of the new agent with standard treatments, these studies also gather more information about the safety and effectiveness in a large group of patients that might involve several hundred to a few thousand people from different populations. These participants might be treated with various dosages of the agent and some with combination of other agents. However, phase III studies are only conducted if the new agent has shown promising potential to become a new standard of care. Finally, the phase IV studies, which are also known as post-marketing surveillance trials, further evaluate the effectiveness and long-term safety of the new agent (Begg et al., 1996; NCI, 2013; US FDA, 2014).

The efficacy of reovirus serotype 3 Dearing strain as therapy has been demonstrated in animal models of many human cancers by intratumoral and intravenous administration (Alain et al., 2002; Hirasawa et al., 2003; Yang et al., 2004). These preclinical studies have provided adequate information to test the feasibility of reovirus in various cancers in the form of

clinical trials. Therefore, the development of Reolysin (reovirus serotype 3 Dearing strain) as a potential cancer therapy has been orchestrated by Oncolytics Biotech Inc. with a total of 32 completed and ongoing clinical trials up-to-date, involving melanoma, sarcoma, multiple myeloma, solid tumor, colorectal, prostate, pancreatic, lung, ovarian, brain, breast, head and neck cancer. These studies included 11 phase I, four phase I/II, 14 phase II, two translational and one phase III clinical trial (www.oncolyticsbiotech.com/clinical-trials).

In the phase I single agent studies with dose escalation, it was reported that reovirus was well tolerated with minimal low grade toxicities and MTD was not achieved, either intratumorally (Forsyth et al., 2008; Morris et al., 2013) or intravenously (Gollamudi et al., 2010; Lolkema et al., 2011; Vidal et al., 2008). The phase II studies were conducted with predetermined treatment regimes and results have shown that reovirus induces prominent anti-tumor efficacy (Galanis et al., 2011). Furthermore, the combination of reovirus and other standard therapy was proven to be well-tolerated with evidence of reovirus activity in the tumors (Comins et al., 2010; Harrington et al., 2010; Kanapanagiotou et al., 2012). The one and only phase III study was on the intravenous reovirus therapy in combination with carboplatin and paclitaxel in head and neck cancers (www.oncolyticsbiotech.com/clinical-trials). This trial was conducted on expanded groups consisting of patients with certain disease conditions and controls. The progression free survival of the patients was greater than expected, with a statistically significant response in patients treated with a combination of reovirus and the chemotherapeutic agents.

As our study of reovirus as an oncolytic virotherapeutic agent is the first in veterinary oncology, supporting data is needed to predict a suitable administration dose of reovirus administration and to foresee the possible toxicities and subsequent viral shedding in canine cancer patients. However, the necessary data was only available for reovirus serotype 1 (Lou and Wenner, 1963; Thompson et al., 1970) but not for reovirus serotype 3 of the Dearing

strain. Hence, subcutaneous injections of reovirus were administered to three healthy dogs to monitor for any adverse events, viral shedding and immune response.

Based on the information in healthy dogs, we conducted a preliminary study in canine patients bearing different kinds of cancers. The treatment regimes and procedures were performed with reference to the clinical trials using reovirus in human. The primary objectives of the preliminary clinical studies were to determine the possible adverse events of reovirus administration in canine cancer patients and to collect preliminary data for the design of a reovirus treatment regime that can be used in future phase I clinical trials. At the same time, data on the pharmacokinetic of viral shedding, the immune response to reovirus administration and the efficacy of reovirus against cancers in immunocompetent canine patients are essential for the establishment of future studies.

MATERIALS AND METHODS

Experimental infection of reovirus in healthy dogs

Four healthy Beagles, aged 1-3 years old, were used for the experimental infection of reovirus which was conducted in Nippon Zenyaku Kogyo Co., Fukushima. They were kept in a clean, sterile environment and fed once a day. Subcutaneous injections of 1.4 x 10^8 $TCID_{50}$ of reovirus diluted in 1 ml normal saline was given to three Beagles for three consecutive days (Table V-1). One additional dog was kept as sentinel in a kennel located in the same enclosed room. General body condition, appetite, excrements and anal temperature were monitored daily. Blood, oral swab, urine and feces were collected at D-1, D0, D1, D2, D3, D5, D7, D10, D14 and D28. Blood was collected from the jugular vein into blood collection serum tubes (BD Vacutainer) before being centrifuged and serum stored for further analysis. All samples were analyzed with real time RT-PCR for the detection of reovirus and only selected serum samples were used for the neutralizing anti-reovirus antibodies (NARA) assay.

Canine cancer patients

All the canine cancer patients in the preliminary study are patients of the Yamaguchi University Animal Medical Center (YUAMEC). Canine cancer patients with full consent from the owners and were healthy enough to withstand reovirus treatment were eligible for the study. Most of the canine patients have undergone other standard therapies with minimum success or were unable to undergo anaesthesia due to advanced age. All clinical procedures were approved by the ethics committee of the Joint Faculty of Veterinary Medicine, Yamaguchi University (Permit Number: 001). Efforts were made to ensure minimal pain and suffering of the patients. Information of patient and disease is summarized in Table V-4.

Study design and dosing scheme

The dosing scheme is shown in Table V-5. As this is the first administration of reovirus in canine cancer patients, a conservative starting dose of 1 x 10^8 $TCID_{50}$ injected intratumorally once was selected for patient 1. Reovirus was given at 1 x 10^8 $TCID_{50}$ intratumorally twice three weeks apart in patient 2 and dose escalation was started in patient 3 whereby 1 x 10^9 and 5 x 10^9 $TCID_{50}$ of reovirus was given intravenously three weeks apart. Patient 4 was only given one intravenous injection of reovirus of 1 x 10^9 $TCID_{50}$. One cycle of 1 x 10^9 $TCID_{50}$ followed by two cycles of 5 x 10^9 $TCID_{50}$ reovirus intravenous injections were given to patient 5 approximately one week apart. The dose and frequency of reovirus administration was determined according to the size of the patient and on the adverse events encountered.

Viral administration, patient monitoring and assessment of adverse events

Purified Reolysin (clinical grade reovirus; GMP) was obtained from Oncolytics Biotech Inc. (Calgary, Canada). Stock were stored at -80˚C and thawed rapidly before diluting into appropriate doses. For intratumoral injections, reovirus was prepared in one ml normal saline and injected only at one site. For intravenous injections, reovirus was diluted in 50 ml normal saline given over two hours in small breed dogs or in 200 ml given over an hour in large breed dogs. Reovirus administration was performed under sterile condition in an isolated room with proper precautions such as using masks, gowns and gloves. Patients were monitored under close observation during the administration procedure and hospitalized throughout the treatment course to monitor for adverse events and viral shedding. Adverse events were graded according to the Veterinary cooperative oncology group- common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or

biological anti-neoplastic therapy in dogs and cats v1.1 (VCOG, 2011). Serum, urine, oral and anal swabs were collected at selected time interval for the detection of viral shedding. NARA titer was also analyzed before and after the administration of reovirus.

Response evaluation

Tumor size was monitored during hospitalization and at subsequent revisits. Tumor in patient 1, 2 and 3 was measured using a caliper at the longest and widest part of the tumor. The esophageal tumor in patient 4 was monitored with computed topography (CT) scan. Ultrasonography was used to measure the size of bladder mass and the internal/ external iliac lymph node of patient 5. Monitoring of the anti-tumor response of reovirus was followed as closely as possible to the Response evaluation criteria for solid tumours in dogs v1.0 by Veterinary Cooperative Oncology Group (Nguyen et al., 2013).

Analysis of viral shedding

Serum, urine, saliva and stool before treatment and throughout the study from reovirus-infected healthy beagles and reovirus-treated canine cancer patients were tested for reovirus particles using reverse transcriptase-polymerase chain reaction (RT-PCR). Only samples from canine cancer patients were co-cultured L929 cell to detect infectious reovirus particles. RNA extraction was performed using ISOGEN-LS (Nippon Gene) and real-time RT-PCR was conducted by Nippon Zenyaku Kogyo Co. (Fukushima) with One Step SYBR Prime Script PLUS RT-PCR Kit (Takara Bio) using primers, 5’-TGGGACAACTTGAGACAGGA-3’ and 5’-CTGAAGTCCACCATTTTGAA-3’ (Decaro et al., 2005), according to the instruction of the manufacturer.

Detection of neutralizing anti-reoviral antibodies (NARA)

Serum was collected and stored at -20˚C for batch analysis of NARA assay as previously reported (White et al., 2008). In brief, L929 cells were plated in 96-well plates at 7,500 cells per well and incubated overnight at 37˚C and 5% CO_2. Reovirus stock was prepared in two dilution series (2- and 10-fold) such that the final dilutions of the two series were 1:204,800 and $1:10^{12}$ before incubation with L929 cells at 37˚C and 5% CO_2 for two hours. The reovirus inoculum was replaced with fresh growth medium and MTT assay performed to measure cell survivability after 48 hours.

As for the measurement of the NARA titer in clinical specimens, the steps were repeated with a constant titer of reovirus (known to cause 80% cell death) that was pre-incubated with 4-fold dilution series of the clinical serum samples. A negative control of dog serum was always included and the serum samples were heat-inactivated at 56˚C for 30 minutes.

RESULTS

Experimental infection of reovirus in healthy dogs

In order to predict the possible adverse events that might arise from the administration of reovirus in dogs, three healthy Beagles were experimentally infected with reovirus for three consecutive days (Table V-1). No adverse events were seen except for one episode of vomiting and diarrhea in one of the infected dogs (Table V-2). Increment of the NARA titer can be seen as early as 7 days post-infection (dpi) for up to 28 days (Table V-3; Fig. V-1). All dogs injected with reovirus had an increment of NARA titer, which was not seen in the sentinel dog. Even though a fairly high titer of reovirus was injected into the dogs, no viral shedding was detected at any time point (Table V-3).

Patient characteristics

Five canine cancer patients (2 males and 3 females) with various types of advanced cancer that were unresponsive to existing standard cancer therapies or were unable to undergo anaesthesia due to advanced age were enrolled into this study. Patient demographics with details of primary tumor diagnosis and prior therapies are shown in Table V-4. The median age of the patients was 10.8 years (range 6-15). Two of the patients were suffering from cutaneous mast cell tumor (MCT) and there was one patient with mammary gland adenocarcinoma, adenocarcinoma of the esophagus and transitional cell carcinoma (TCC) of the urinary bladder, respectively. All patients had prior therapies such as radiotherapy, surgical resection or chemo/ medical therapy before reovirus treatment except for patient 2 and 4 who were unable to undergo anaesthesia. All patients also had metastasis to regional lymph nodes before the administration of reovirus except patient 4.

Dosing Scheme

The reovirus dosing scheme and other concurrent treatment is summarized in Table V-5. Patient 1 received one 1 x 10^8 $TCID_{50}$ of reovirus intratumorally while patient 2 received one 1 x 10^8 $TCID_{50}$ of reovirus intratumorally for cycle 1 and three reovirus of the same dose intratumorally for three consecutive days for cycle 2. As the reovirus-induced toxicities in patient 1 and 2 were mild, the amount of reovirus in patient 3 was increased to 1 x 10^9 $TCID_{50}$ for three consecutive intravenous injections. Inter-patient dose escalation to 5 x 10^9 $TCID_{50}$ for 5 consecutive days was carried out in patient 3 for the following cycle. Patient 3 also had concurrent treatment of piroxicam and cyclophosphamide. Only one cycle of five intravenous injections of 1 x 10^9 $TCID_{50}$ in combination with cyclophosphamide was given to patient 4. Patient 5 received five consecutive intravenous injections of reovirus at 1 x 10^9 $TCID_{50}$ at the first cycle and one 5 x 10^9 $TCID_{50}$ of intravenous injection every week thereafter. Patient 5 was treated concurrently with gemcitabine and the combination treatment of reovirus and gemcitabine is still ongoing.

Adverse events

Reovirus treatment was well-tolerated in all the canine cancer patients and all symptomatic toxicities encountered (either definitely or probably related to viral administration) were mild (≤ grade 2; Table V-6). The most observed adverse events, especially during the first few days after reovirus injection, were lethargy, fever, anorexia, diarrhea, vomiting and tumor pain.

Among the complete blood count (CBC) and blood biochemistry parameters that were examined, only the elevation of C-reactive protein (CRP) seemed to be related to reovirus administration (Fig. V-2). The increment of CRP overlaped with the occurrence of fever, the gastrointestinal symptoms and tumor pain. Liver and kideny tests remained normal after

reovirus treatment. Bleeding and inflammation of the cutaneous MCT a few days after reovirus intratumoral injection, which cause a bearable amount of pain, was observed in both patient 1 and 2. The swelling of the right hindleg of patient 3 after reovirus treatment was not likely to be related to the reovirus injection but most probably due to obstructive lymphadenopathy.

NARA response

All patients were tested before the start of reovirus treatment for NARA titer and all the five patients had a low titer, ranging from 16 to 256 (Table V-7). All of the patients became seropositive as early as 7 dpi with titers reaching as high as 262,144 at the end of the first treatment cycle. Subsequent cycles of reovirus treatment in patient 2, 3 and 5 continued to stimulate antibody production, reaching a peak of 4,194,304 in patient 5 (Fig. V-3).

Pharmacokinetics analysis of viral shedding

Viral shedding was examined by RT-PCR in serum, urine, saliva and stool. Viral RNA was amplified in at least one of the samples taken from all the patients except in patient 4 (Table V-7). Viral shedding from the saliva and stool were detected from both the cutaneous MCT patients. Viral particles were also detected from the serum of patient 1, 2, 3 and 5 while urine from patient 2, 3 and 5 was positive for reovirus particles. The co-culture of serum and urine from patient 5 with L929 cells were the only samples that induced cytopathic effects (CPE).

Anti-tumor response assessment

Even though assessment of the efficacy of reovirus was not our objective in this study, the anti-tumor response was still documented at the end of hospitalization and at as many

subsequent hospital revisits as possible. The cutaneous MCT, as well as the regional lymph nodes in patient 1 increased in size progressively until day 16 post-reovirus administration (Fig. V-4). Even though the size of the cutaneous MCT in patient 2 reduced slightly at day 14 post-treatment of cycle 1 (Fig. V-5), the tumor increased in size when measured at day 24 of cycle 2 (Fig. V-6). Assessment of the diffused mammary mass in patient 3 was particularly challenging due to the irregularity and diffused nature of the cancer. Measurements indicated that there was no difference in size before and after the first and second cycle of treatment (Fig. V-7 and V-8). Evaluation of the esophageal mass in patient 4 was performed by CT scan due to the nature of the cancer (Fig. V-9). However, the condition of this patient deteriorated and was withdrawn from the study. Therefore, the evaluation of patient 4 ended at day 7 of one cycle of reovirus treatment. The TCC of the urinary bladder in patient 5 was monitored by ultrasonography performed by the same examiner throughout the study (Fig. V-10). The size of the tumor and regional lymph node reduced after 3 cycles of treatment and treatment is still ongoing for this patient.

DISCUSSION

Reovirus is found ubiquitously and can virtually infect all kinds of mammals, including humans and dogs (Rosen, 1962). The results in Chapter 4 have indicated that approximately 50% of the healthy canine population has been naturally infected with reovirus serotype 1, 2 or 3. Up to date, there are only reports on the experimental infection of reovirus serotype 1 in healthy puppies (Lou and Wenner, 1963; Thompson et al., 1970) but none on the infection of reovirus serotype 3 Dearing in healthy dogs. Therefore, in the first part of this chapter, experimental infection of reovirus in healthy dogs was assessed by three consecutive subcutaneous injections of 1 x 10^8 $TCID_{50}$ of reovirus in beagles to determine the possible adverse events and viral shedding. The immune response against reovirus infection in each of the infected dogs was also compared to a sentinel dog kept in the same environment.

Among the three infected dogs, the only adverse events observed were one episode of vomiting and diarrhea in one of the dogs (Table V-2). There was no elevation of the NARA titer in the sentinel dog (Table V-3; Fig. V-1) kept in the same environment as the infected dogs. However, elevation of NARA titer was detected as early as 7 days post-injection and as high as 4,096 fold increment compared to the NARA baseline titer (Table V-3; Fig. V-1). None of the serum, urine, saliva and fecal samples was tested positive for the reovirus RNA (Table V-3). Judging from the lack of adverse events and viral shedding, reovirus treatment seems like a plausible option in canine cancer patients as the immune system can overcome the deliberate infection and prevent clinical manifectation.

Armed with these data from healthy dogs, we moved on to the preliminary studies of reovirus treatment involving five canine cancer patients highlighted in the second part of this chapter. The main objectives were to evaluate the possible adverse events in canine cancer patients at various reovirus dosing schemes and to collect first hand information for the design of a reovirus treatment regime for future phase I clinical trials. Assessment of viral

shedding, anti-viral and anti-tumor response after reovirus treatment were also carried out at the same time to facilitate future studies.

The starting dose of reovirus administered was 1 x 10^8 $TCID_{50}$ in the first patient and was increased to a maximum of 5 x 10^9 $TCID_{50}$ in the fifth patient (Table V-5). At these doses, the adverse events that were observed were lethargy/ fatigue/ inactivity, fever, weight loss, anorexia, vomiting, diarrhea and pain at tumor site (Table V-6). These adverse events were mild and no interventional therapy was required. In human clinical studies, the MTD has not been reached even at a reovirus dose as high as 1 x 10^{10} $TCID_{50}$ given intratumorally (Forsyth et al., 2008; Harrington et al., 2010; Morris et al., 2013) and 3 x 10^{10} $TCID_{50}$ given intravenously (Vidal et al., 2008; Gollamudi et al., 2010). Adverse events reported in these human clinical trials were nausea, vomiting, diarrhea, injection site erythema, fever/ chills, flu-like illness and arthralgia/ myalgia, which were similar to those in our study. Therefore, in future clinical studies involving canine cancer patients, it is still possible to increase the dose of reovirus, either to be administered intratumorally or intravenously, perhaps as high as or higher than that of human clinical trials.

Both intratumoral and intravenous administration of reovirus has been tested in human clinical trials. The intratumoral route of administration was chosen for patient 1 and 2 with MCT in order to assess the effects of direct administration of reovirus into the tumor mass. Reovirus at 1 x 10^8 $TCID_{50}$ in one ml normal saline was injected intratumorally only at one site. The effects of reovirus were dramatic during the first few days after virus injection but quickly disappeared and the tumor continued to grow larger. One improvement strategy that can possibly be applied for the intratumoral administration in the future is to divide the total dose of reovirus so that equal amount per one cm^2 can adequately penetrate the whole lesion (Morris et al., 2013). This strategy will allow the whole tumor mass to be infected by reovirus at the same time, in hope that direct oncolysis will take place in the whole tumor bulk, which

can prevent any resistance to reovirus or regrowth of the tumor. The intravenous route was chosen for patient 3, 4 and 5 as intravenous administration of reovirus is effective in targeting disseminated or unapproachable tumors. Studies have shown that reovirus can localize in susceptible tumors after systemic delivery (Vidal et al., 2008; Galanis et al., 2012). However, the possibility of the anti-viral immune response limiting the efficacy reovirus is higher with systemic delivery of reovirus as compared to intratumoral administration (Coffey et al., 1998; White et al., 2008), suggesting that repeated higher doses of reovirus should be used for intravenous delivery.

Among all the blood parameters that were monitored, only the elevation of C-reactive protein (CRP) seems to be related with the effects of reovirus, where a sharp increase was seen a couple of days after reovirus treatment (Fig. V-2). CRP is one of the major acute phase proteins, where it is used as nonspecific markers of inflammation in dogs (Ceron et al., 2005). The CRP level was at the maximum when lytic effects of the tumors and adverse events were observed, particularly in the MCT patients. Since reovirus infection is known to stimulate an inflammatory response in tumor cells (Errington et al., 2008), we propose that CRP can be used as a biological marker to determine the susceptibility of tumors towards reovirus in a clinical setting. The marker can be included in future clinical studies as an additional assessment criterion of the efficacy of reovirus instead of just depending on the evaluation of tumor size.

RT-PCR and viral culture techniques were used to analyze the pharmacokinetic of reovirus in this study (Table V-7). At least one of the samples from all of the patients except patient 4 was positive for RT-PCR detection. In spite of that, viral culture was only positive for patient 5 in the serum and urine samples. The detection of reovirus was always at early time point after reovirus treatment, which correlated with some of the reovirus toxicities recorded. In human studies, viral detection in bodily fluids was infrequent. Reasons might be

that the reovirus detection are carried out less frequently (weekly) with less extensive RT-PCR cycles (25 or 35) (Morris et al., 2013; Vidal et al., 2008). In this study, bodily fluids were collected daily during treatment through the whole duration of hospitalization and positive samples are usually those during reovirus treatment. 45 RT-PCR cycles were also applied in our study that might lead to a more sensitive viral detection. On top of that, especially in the saliva samples of patient 1 and 2, there stands a possibility of viral contamination due to self-grooming around the tumor .

All the canine patients had a low NARA baseline titer and elevation of the NARA titer was observed after reovirus treatment (Table V-7 and Fig. V-3). The highest elevation of NARA titer was in patient 1 where there was a 16,384 fold increment after one intratumoral injection of 1 x 10^8 $TCID_{50}$ of reovirus, most probably due to the patient being naïve to reovirus infection before treatment. In patient 2, 3 and 5, the NARA titer remained high before subsequent treatment cycle(s) and the titer increased further after reovirus was injected, irrespective of the route of virus administration. This phenomenon involving the anti-viral response was consistent with human studies (Morris et al., 2013). The mild adverse events observed in all the five canine patients support the theory that the elevation of NARA titer plays a protective role against the reovirus infection. This, in turn, might act as an obstacle to the efficient delivery of reovirus to the tumors, especially in cases where the intravenous route was used. Thus, it seems more feasible to combine reovirus and other standard therapy such as chemotherapy, that will attenuate the anti-viral response and enhance the anti-tumoral response. Combination of reovirus and chemotherapy such as cyclophosphamide (Qiao et al., 2008) or radiotherapy (Harrington et al., 2010) has also shown promising results as synergistic agents. Various phase I and II clinical trials have reported the positive results of combination therapy using docetaxel (Comins et al., 2010), carboplatin and paclitaxel (Karapanagiotou et al., 2012) and gemcitabine (Lolkema et al., 2011).

Since the primary objective of this study was not to evaluate the anti-tumor activity of reovirus, five canine patients of various tumor types, aggressiveness of the cancer and previous treatment regimes were recruited in this study. Patient 1 to 4 were loss to follow up after 16 days, 45 days, 26 days and 7 days of participating in this study, due to the lack of compliance of the owners or the death of the patients unrelated to reovirus treatment. According to the guidelines of cRECIST v1.0 by the Veterinary Cooperative Oncology Group (VCOG) (Nguyen et al., 2013), the best overall response for solid tumors should be assessed not less than six to eight weeks after the start of therapy. This highlighted the importance of choosing the optimum eligibility criteria in future studies in order to follow up on the progress of the anti-tumoral response in the participating patients for at least 6 weeks. Only patient 2 fulfilled the minimum 6 weeks of study participation with an overall response of progressive disease. Since patient 5 is still undergoing reovirus treatment, assessment of the anti-tumor activity for this patient will be conducted at the end of six weeks of the study.

Additional assessment criteria should be included into future clinical studies in canine cancer patients. Among these are the histological evaluation and detection of reovirus proteins from biopsy samples of the targeted tumor. Since the evaluation of tumor size is challenging, especially in cases of diffused or irregular tumor mass, biopsy samples will serve as an important indicator of reovirus efficacy in the tumor cells. Besides that, there were some points in this study that can be improved for future studies, such as the different time interval between cycles of reovirus administration and the concomittant usage of other therapies. It is important to decide on an optimal interval between cycles of reovirus treatment as intervals too long might allow tumors a chance to recur and grow. On the other hand, intervals between reovirus treatment that are too short might induce some persistently infected tumor cells that will be resistant to reovirus (Alain et al., 2006; Kim et al., 2007). The combination effects of reovirus with piroxicam, cyclophosphamide and gemcitabine

were also unable to be assessed in this study as limited by the low number of cases and the loss of patient to follow up. Instead, the selection of the best combination therapy should be performed beforehand via *in vitro* and *in vivo* studies before the involvement of actual patients in clinical studies.

The invaluable experience learnt and data collected from these preliminary clinical trials in five canine cancer patients has allowed us to plan for the phase I clinical trial of reovirus treatment in canine cancer patients that will be carried out in the near future. The eligibility criteria of participant, dose elevation scheme, route and interval of reovirus administration, monitoring of adverse events and assessment of the anti-tumor response of reovirus in biopsied tumor samples are the key points of the study that has to be pre-determined wisely. With the objectives of determining the MTD and the tolerance to reovirus, a minimum of 20 canine cancer patients will be recruited for phase I trials in the near future.

TABLE

Table V-1: Characteristics and dosing scheme of healthy dogs

Dog	Sex	Age (y)	Dose ($TCID_{50}$[c])	Route of administration	No. of injections
A	IF[a]	2	Not treated	-	-
B	IF	3	1.4×10^8	Subcutaneous	3 consecutive days
C	IM[b]	1	1.4×10^8	Subcutaneous	3 consecutive days
D	IM	1	1.4×10^8	Subcutaneous	3 consecutive days

[a] Intact female

[b] Intact male

[c] 50% tissue culture infectious dose

Table V-2: Adverse events related to reovirus treatment in healthy dogs

Adverse events	Lethargy/ fatigue/ general performance	Fever	Weight loss	Anorexia	Diarrhea	Vomiting
Dog A	-	-	-	-	-	-
Dog B	-	-	-	-	Grade 1	Grade 1
Dog C	-	-	-	-	-	-
Dog D	-	-	-	-	-	-

Table V-3: NARA response and pharmacokinetics of viral shedding in healthy dogs

Dog	Dose ($TCID_{50}$[a])	NARA baseline titer	NARA max titer	RT-PCR serum	RT-PCR saliva	RT-PCR urine	RT-PCR feces
A	Not treated	256	256	Neg	Neg	Neg	Neg
B	1.4×10^8	16	4,096	Neg	Neg	Neg	Neg
C	1.4×10^8	16	65,536	Neg	Neg	Neg	Neg
D	1.4×10^8	256	16,384	Neg	Neg	Neg	Neg

[a] 50% tissue culture infectious dose

Table V-4: Characteristics of canine patients

Patient No.	Breed	Age (y)	Sex	Diagnosis	Location of metastases	Prior therapy
1	Maltese	6	CM[a]	Cutaneous mast cell tumor (MCT)	Submandibular and prescapular l/n[e]	Radiation therapy
2	Toy poodle	15	IF[b]	Cutaneous mast cell tumor (MCT)	Submandibular, prescapular, popliteal l/n	-
3	Dalmatian	13	SF[c]	Mammary gland adenocarcinoma	Popliteal l/n	Surgical resection, radiation therapy
4	Shih Tzu	11	IM[d]	Adenocarcinoma of esophagus	-	-
5	Toy poodle	9	IF	Transitional cell carcinoma (TCC) of the urinary bladder	Int./ ext. iliac l/n	Surgical resection, chemo/ medical treatment

[a] Castrated male
[b] Intact female
[c] Spayed female
[d] Intact male
[e] Lymph node

Table V-5: Dosing scheme in canine patients

Patient No.	Cycle	Dose ($TCID_{50}$[a])	Route of administration	No. of injections	Frequency of injections	Concurrent therapy
1	1	1×10^8	Intratumoral	1	-	-
2	1	1×10^8	Intratumoral	1	3 weeks	-
	2	1×10^8	Intratumoral	3 (consecutive days)	-	-
3	1	1×10^9	Intravenous	3 (consecutive days)	3 weeks	Piroxicam, cyclophosphamide
	2	5×10^9	Intravenous	5 (consecutive days)	-	Piroxicam, cyclophosphamide
4	1	1×10^9	Intravenous	5 (consecutive days)	-	Cyclophosphamide
5	1	1×10^9	Intravenous	5 (consecutive days)	1 week	-
	2	5×10^9	Intravenous	1	1 week	Gemcitabine
	3	5×10^9	Intravenous	1	10 days	Gemcitabine

[a] 50% tissue culture infectious dose

Table V-6: Adverse events related to reovirus treatment in canine patients

Adverse events	Lethargy/ fatigue/ general performance	Fever	Weight loss	Anorexia	Diarrhea	Vomiting	C-reactive protein (CRP)	Tumor pain
None	Patient 3, 4, 5	Patient 2, 5	Patient 3, 4	Patient 3, 4, 5	Patient 4	Patient 4, 5	-	Patient 4, 5
Grade 1	Patient 1	Patient 1	Patient 1, 2, 5	Patient 1	Patient 2, 3, 5	Patient 1, 2, 3	Patient 3	Patient 1, 3
Grade 2	Patient 2	Patient 3, 4	-	Patient 2	Patient 1	-	Patient 1, 2, 4, 5	Patient 2
Grade 3	-	-	-	-	-	-	-	-
Grade 4	-	-	-	-	-	-	-	-

Table V-7: NARA response and pharmacokinetics of viral shedding in canine patients

Patient No.	Cycle	Dose ($TCID_{50}$)	NARA baseline titer	NARA max titer	RT-PCR serum	RT-PCR saliva	RT-PCR urine	RT-PCR feces	Viral culture
1	1	1×10^8	16	262,144	D1	D1, 3, 7, 9, 11	Neg	D9, 13, 14	Neg[a]
2	1	1×10^8	256	262,144	Neg	D4, 5, 6	D5, 7	D6, 7	Neg[b]
	2	1×10^8	65,536	262,144	D4	Neg	Neg	Neg	Neg[a]
3	1	1×10^9	64	16,384	D2, 3, 4	Neg	D2	Neg	Neg[a, b]
	2	5×10^9	16,384	65,536	Neg	Neg	Neg	Neg	Neg
4	1	1×10^9	256	65,536	Neg	Neg	Neg	Neg	Neg
5	1	1×10^9	64	262,144	D2, 3, 4, 5, 6	Neg	D3	Neg	Serum, urine
	2	5×10^9	-	1,048,576	Neg	Neg	Neg	Neg	Serum
	3	5×10^9	-	4,194,304	Neg	Neg	Neg	Neg	Serum[c]

[a] Only performed with serum samples

[b] Only performed with urine samples

[c] Not performed with urine samples

FIGURE

Figure 1

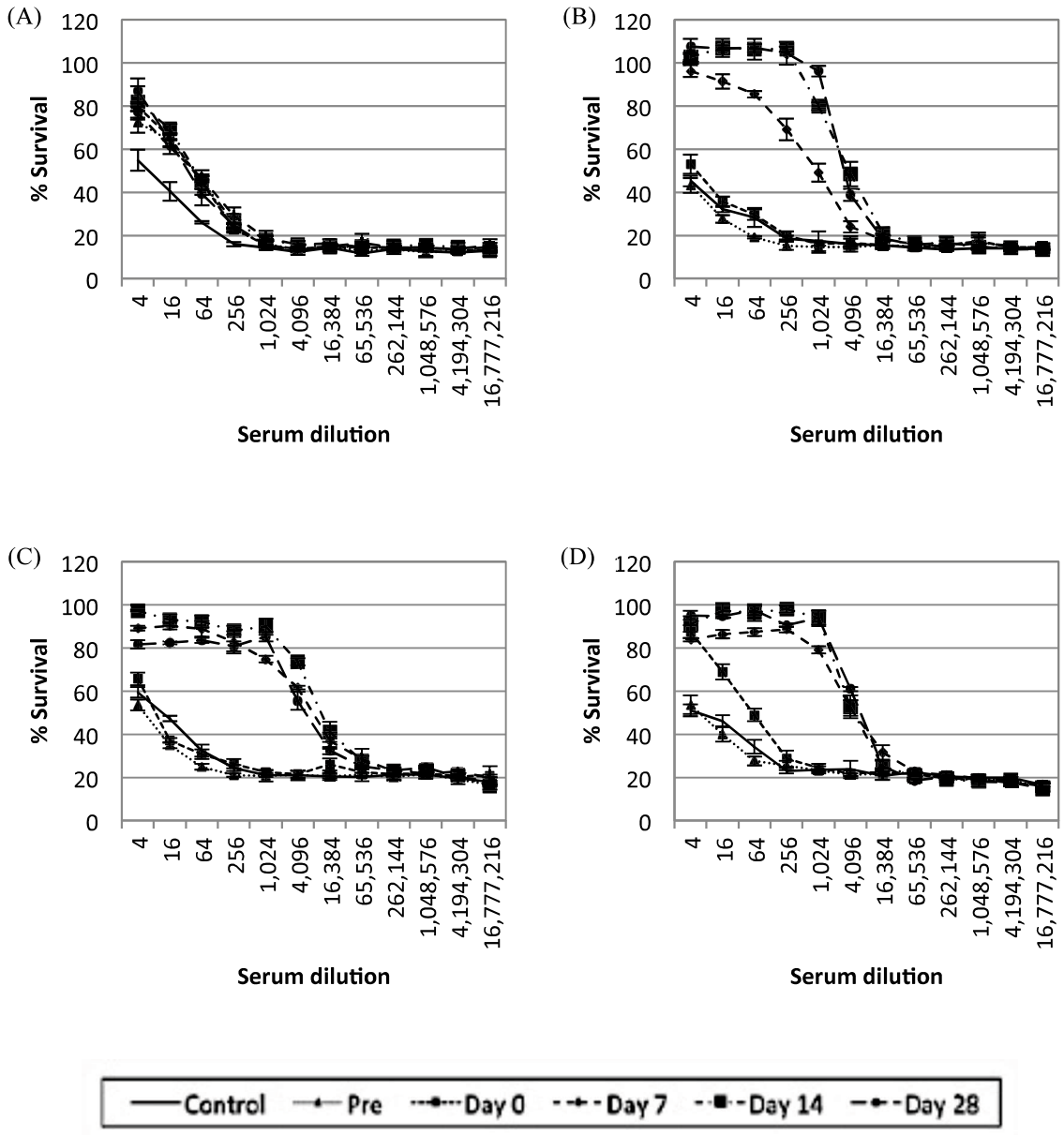
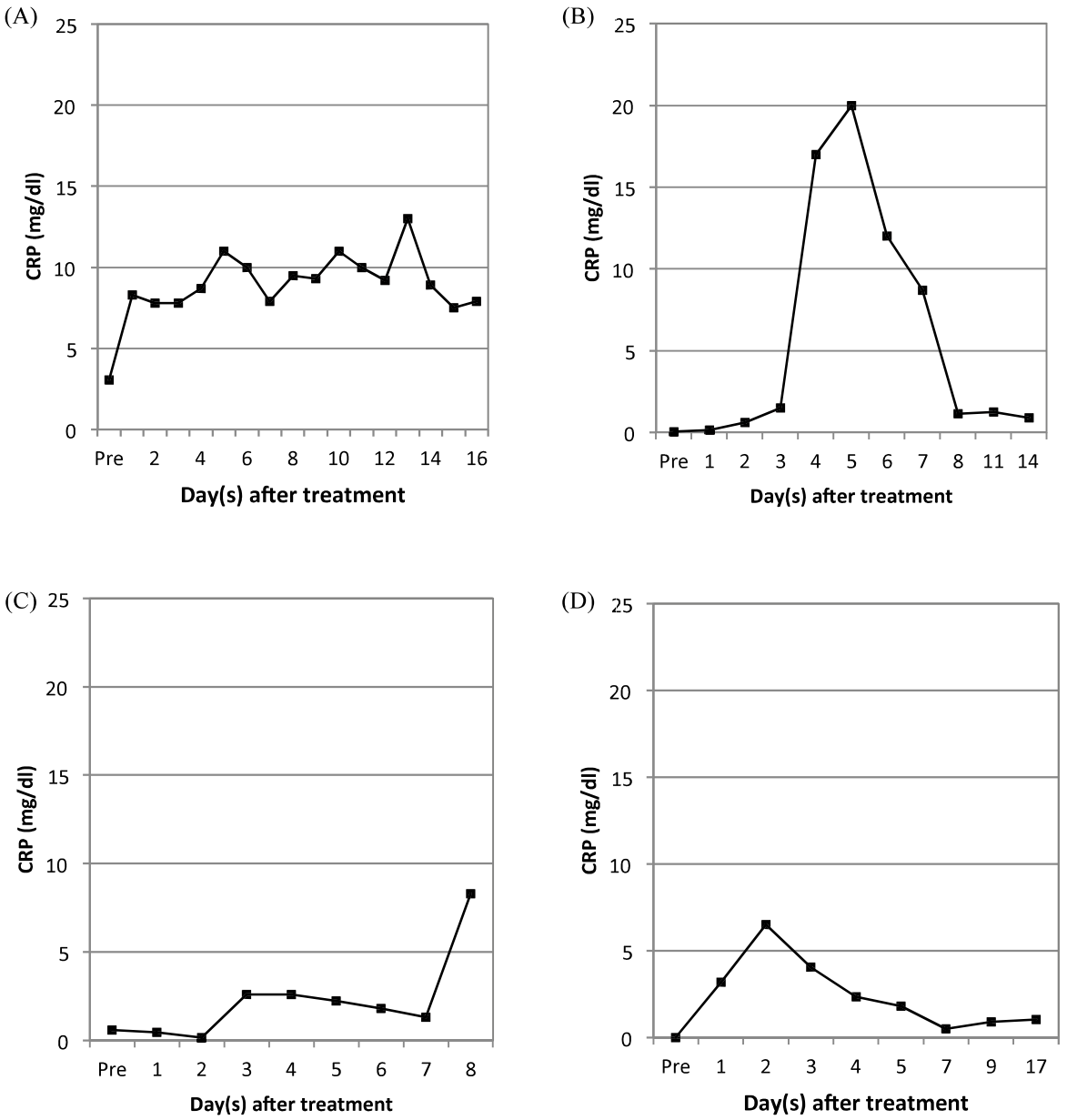


Fig. V-1: NARA assay in healthy dogs. (A) Dog A (sentinel). (B) Dog B. (C) Dog C. (D) Dog D. MTT assay was performed and the percentage of cells that resisted reovirus-induced cell killing was expressed as % survival. In each graph, the control curve represents data obtained using serum from a healthy dog. Mean ± s.e.m., n = 3.

Figure 2


(A)
CRP (mg/dl)
0
5
10
15
20
25
Pre 2 4 6 8 10 12 14 16
Day(s) after treatment
(B)
CRP (mg/dl)
0
5
10
15
20
25
Pre 1 2 3 4 5 6 7 8 11 14
Day(s) after treatment
(C)
CRP (mg/dl)
0
5
10
15
20
25
Pre 1 2 3 4 5 6 7 8
Day(s) after treatment
(D)
CRP (mg/dl)
0
5
10
15
20
25
Pre 1 2 3 4 5 7 9 17
Day(s) after treatment

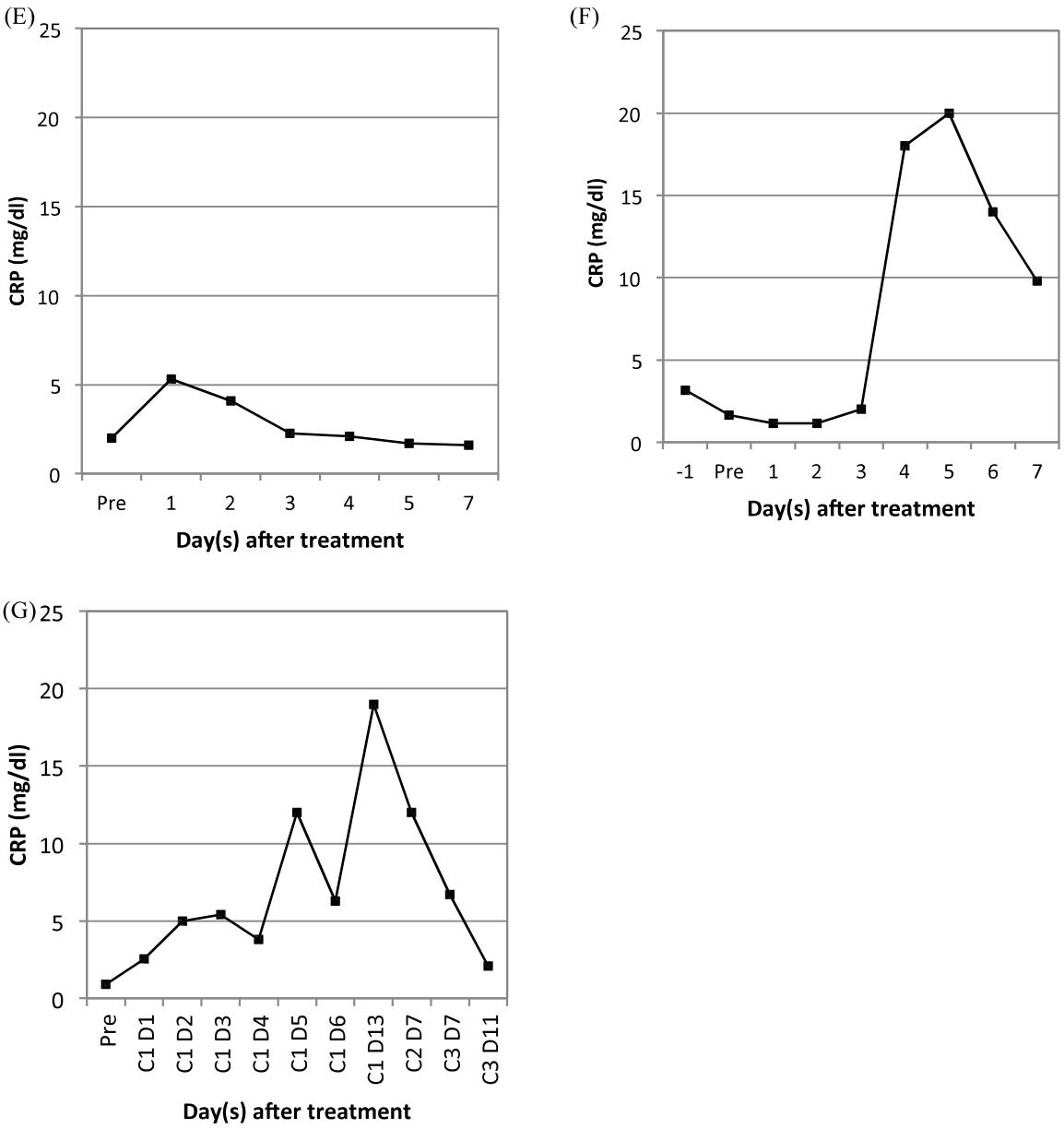
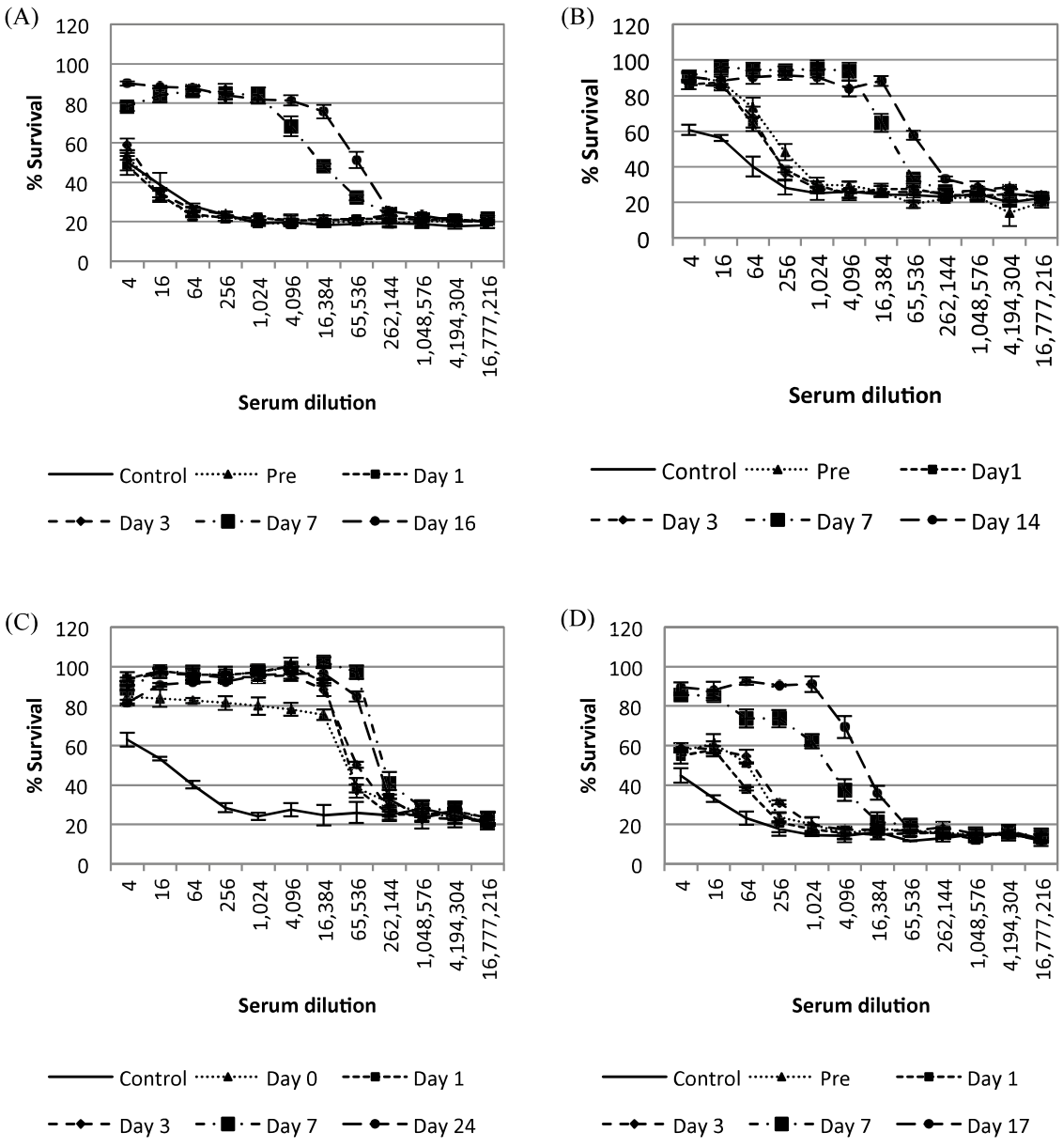


Fig. V-2: CRP levels in canine cancer patients. (A) Patient 1 (B) Patient 2 cycle 1 (C) Patient 2 cycle 2 (D) Patient 3 cycle 1 (E) Patient 3 cycle 2 (F) Patient 4 (G) Patient 5.

Figure 3


(A)
% Survival
120
100
80
60
40
20
0
4
16
64
256
1,024
4,096
16,384
65,536
262,144
1,048,576
4,194,304
16,777,216
Serum dilution
Control
Pre
Day 1
Day 3
Day 7
Day 16
(B)
% Survival
Serum dilution
Control
Pre
Day1
Day 3
Day 7
Day 14
(C)
% Survival
Serum dilution
Control
Day 0
Day 1
Day 3
Day 7
Day 24
(D)
% Survival
Serum dilution
Control
Pre
Day 1
Day 3
Day 7
Day 17

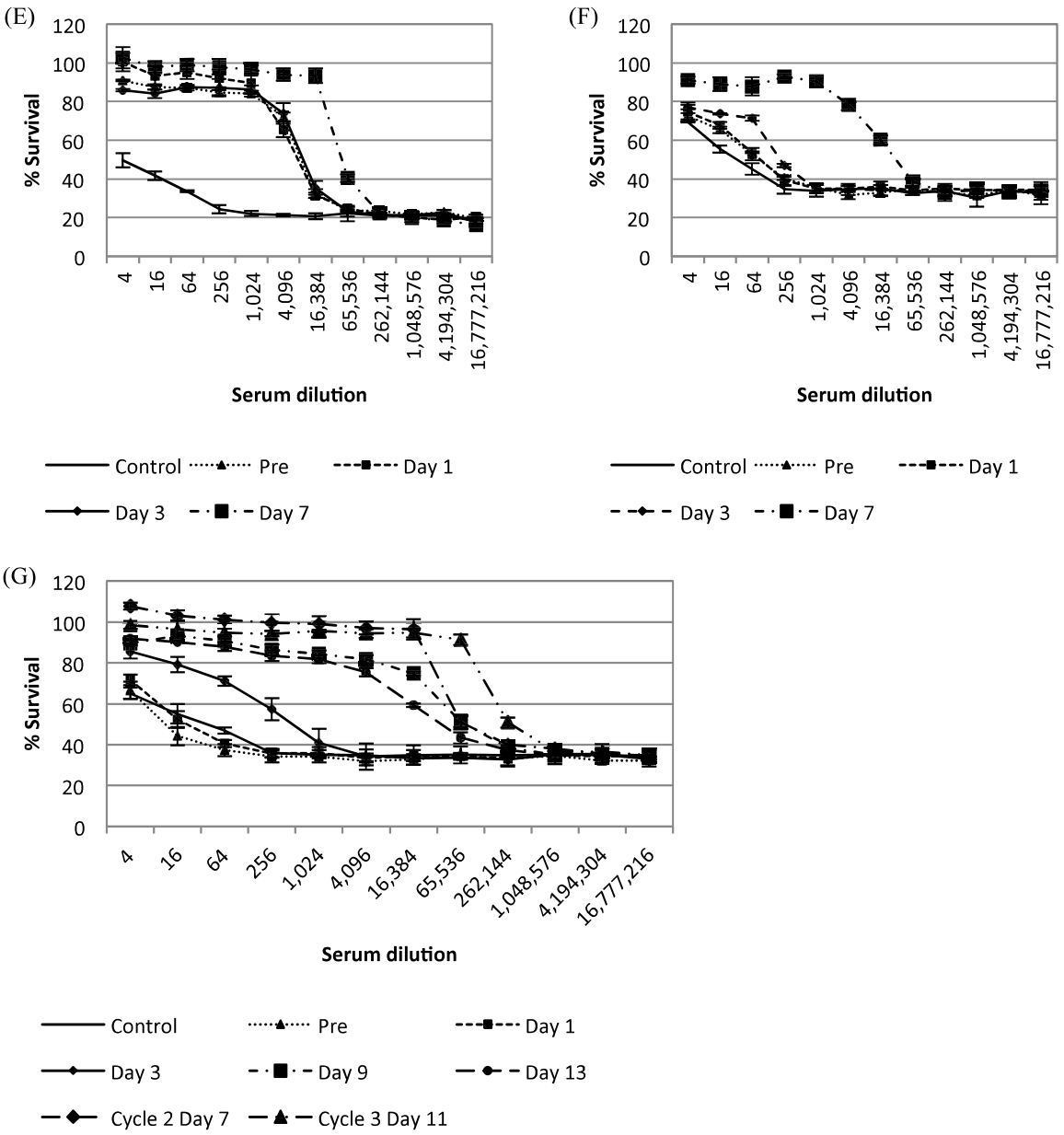


Fig. V-3: NARA assay in canine cancer patients. (A) Patient 1 (B) Patient 2 cycle 1 (C) Patient 2 cycle 2 (D) Patient 3 cycle 1 (E) Patient 3 cycle 2 (F) Patient 4 (G) Patient 5. MTT assay was performed and the percentage of cells that resisted reovirus-induced cell killing was expressed as % survival. In each graph, the control curve represents data obtained using serum from a healthy dog. Mean ± s.e.m., n = 3.

Figure 4

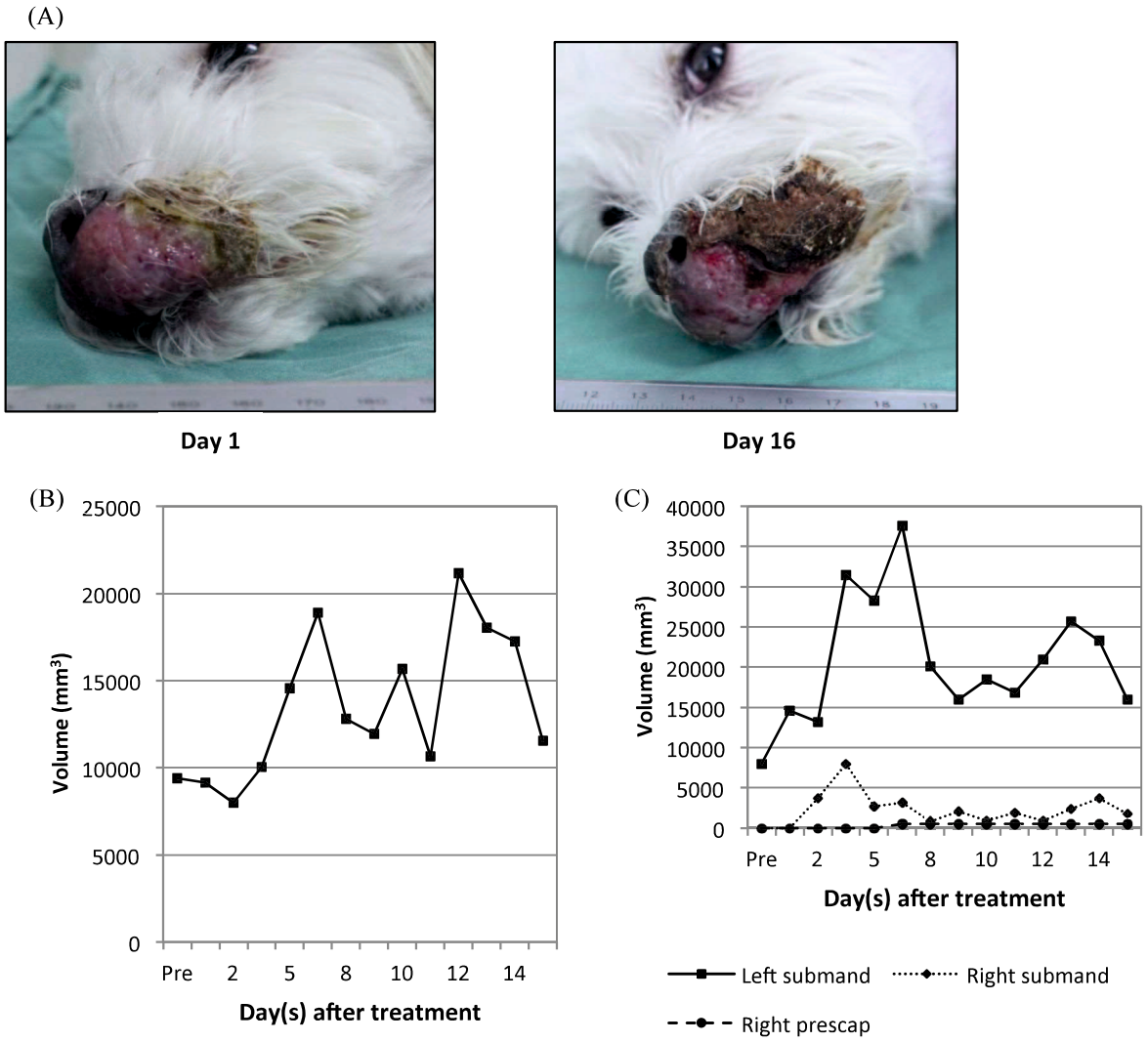


Fig. V-4: Patient 1. (A) Pictures of MCT mass at day 1 and day 16 post-treatment. (B) Graph representing size of MCT mass throughout hospitalization. (C) Graph representing the size of lymph nodes involved.

Figure 5

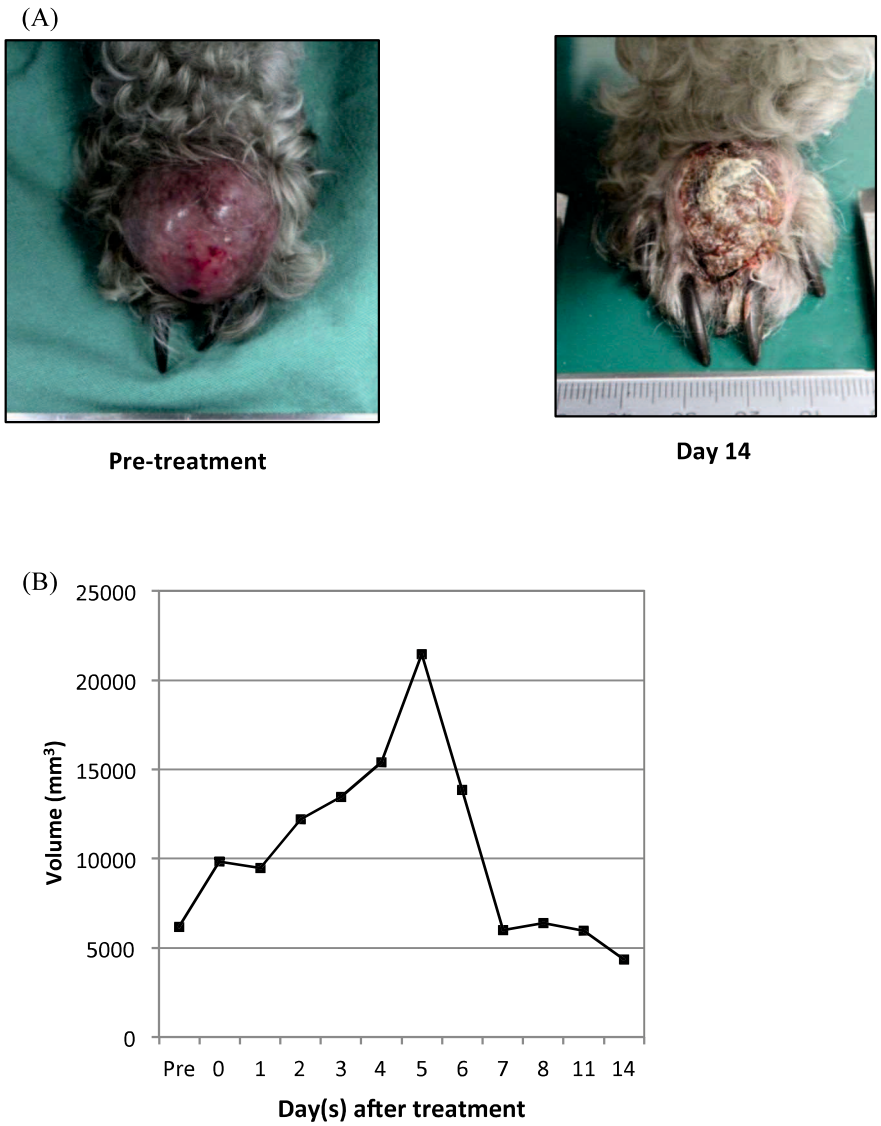


Fig. V-5: Patient 2 reovirus treatment cycle 1. (A) Pictures of MCT mass at pre-treatment and day 14 post-treatment. (B) Graph representing size of MCT mass in cycle 1.

Figure 6

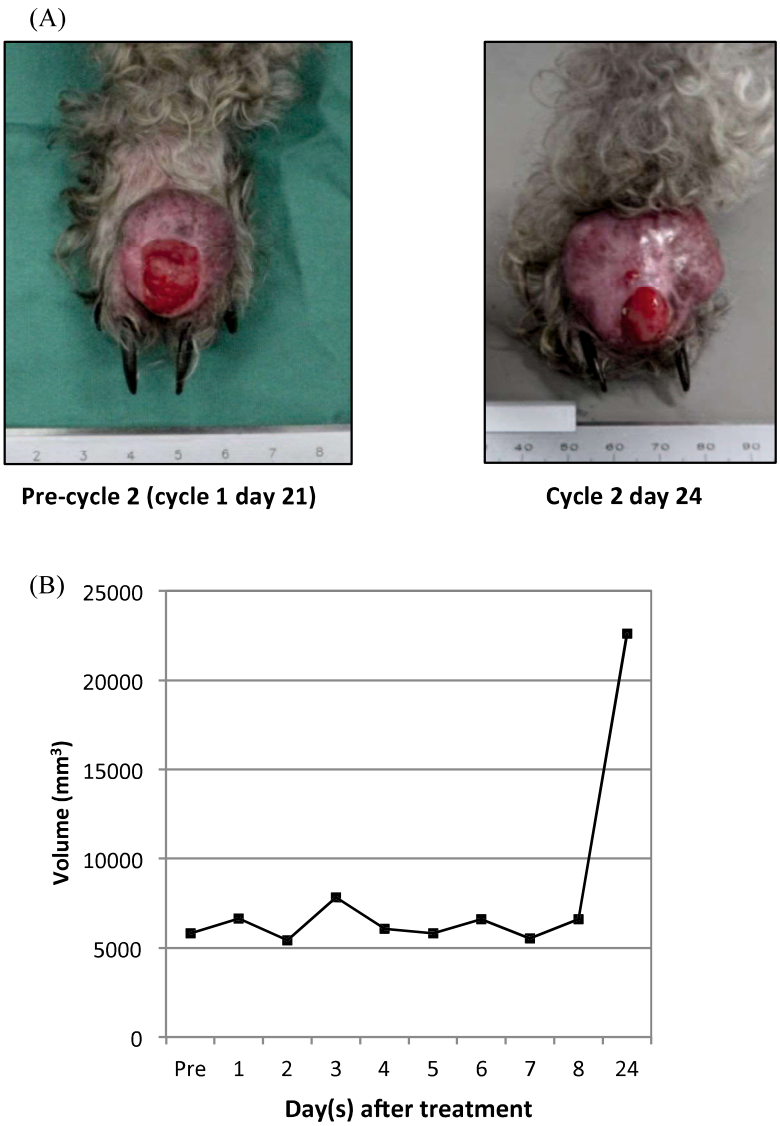


Fig. V-6: Patient 2 reovirus treatment cycle 2. (A) Pictures of MCT mass at pre-treatment of cycle 2 (cycle 1 day 21 post-treatment) and cycle 2 day 24 post-treatment. (B) Graph representing size of MCT mass in cycle 2.

Figure 7

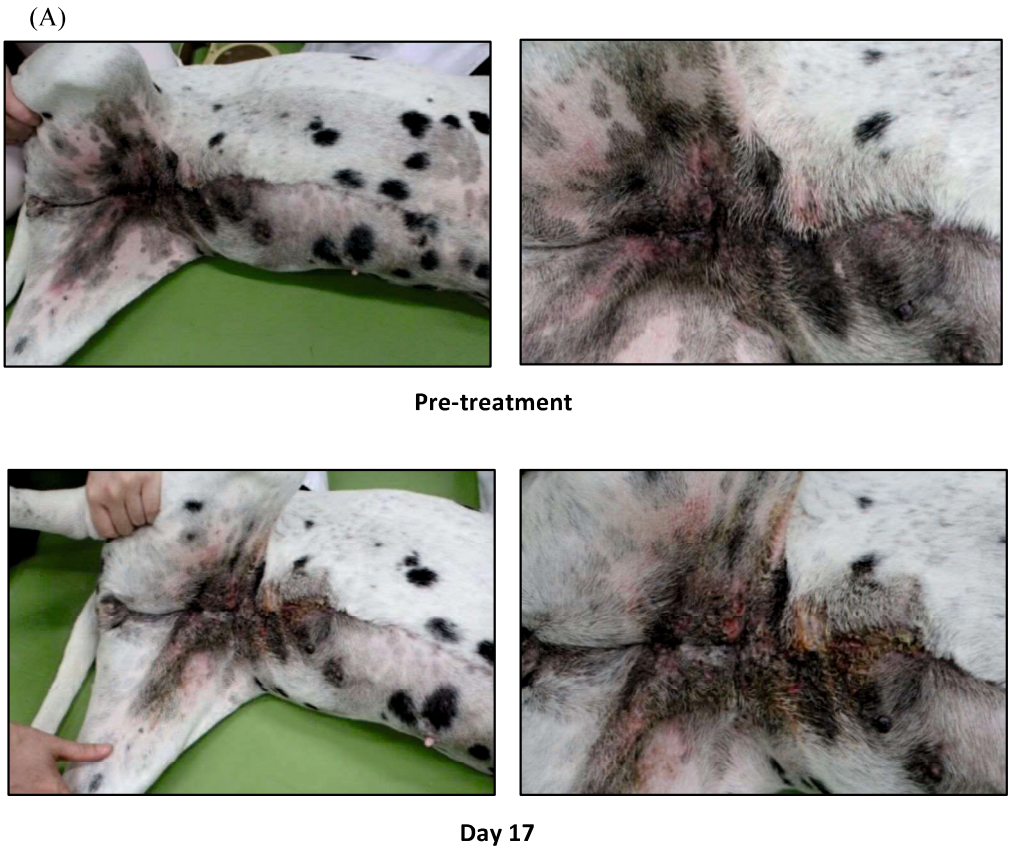
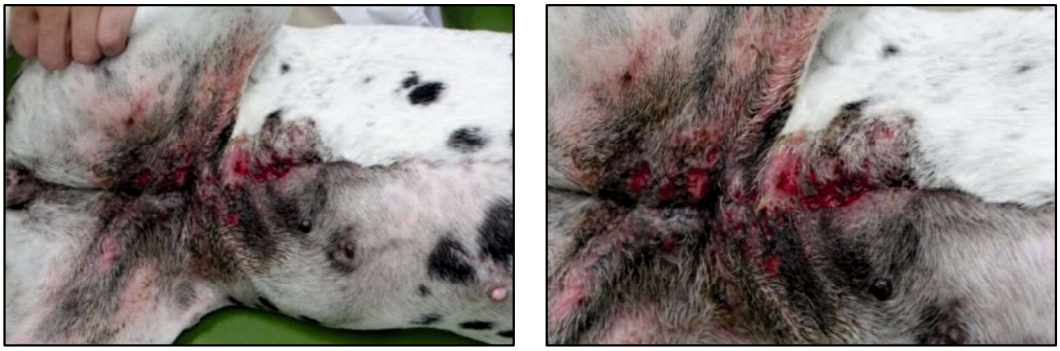


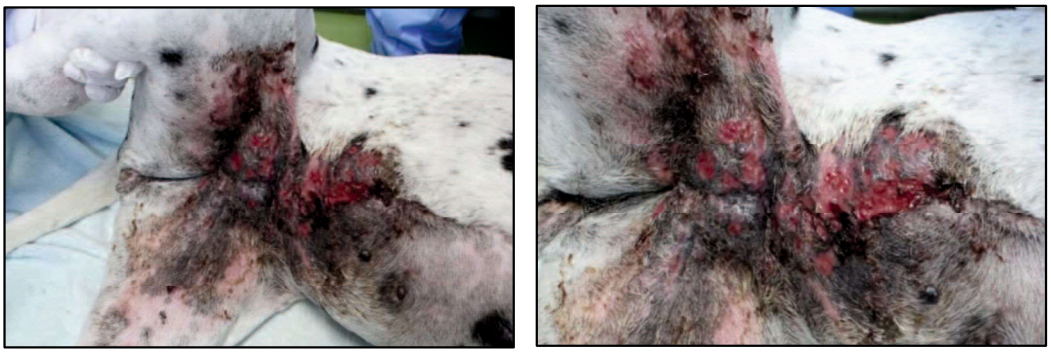
Fig. V-7: Patient 3 reovirus treatment cycle 1. (A) Pictures of mammary mass at pre-treatment and day 17 post-treatment. (B) Graph representing measurement of mammary mass during cycle 1 of reovirus treatment.

Figure 8

(A)



Pre-cycle 2 (Cycle 1 day 23)



Cycle 2 day 7

(B)

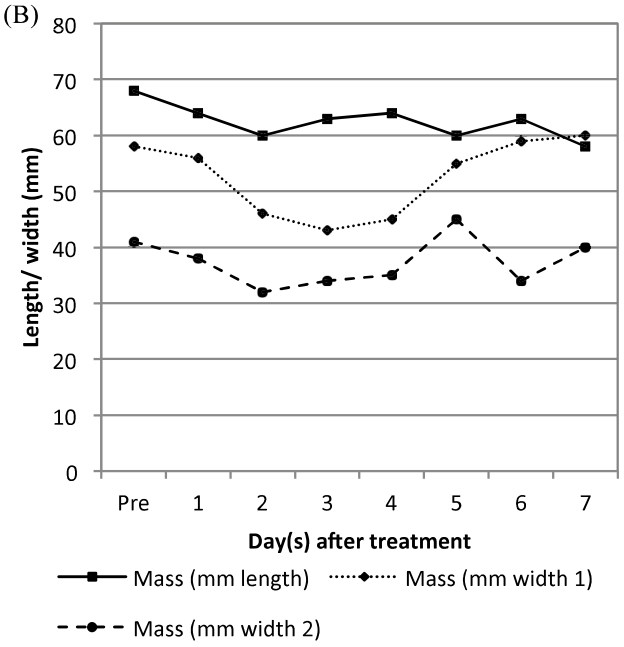
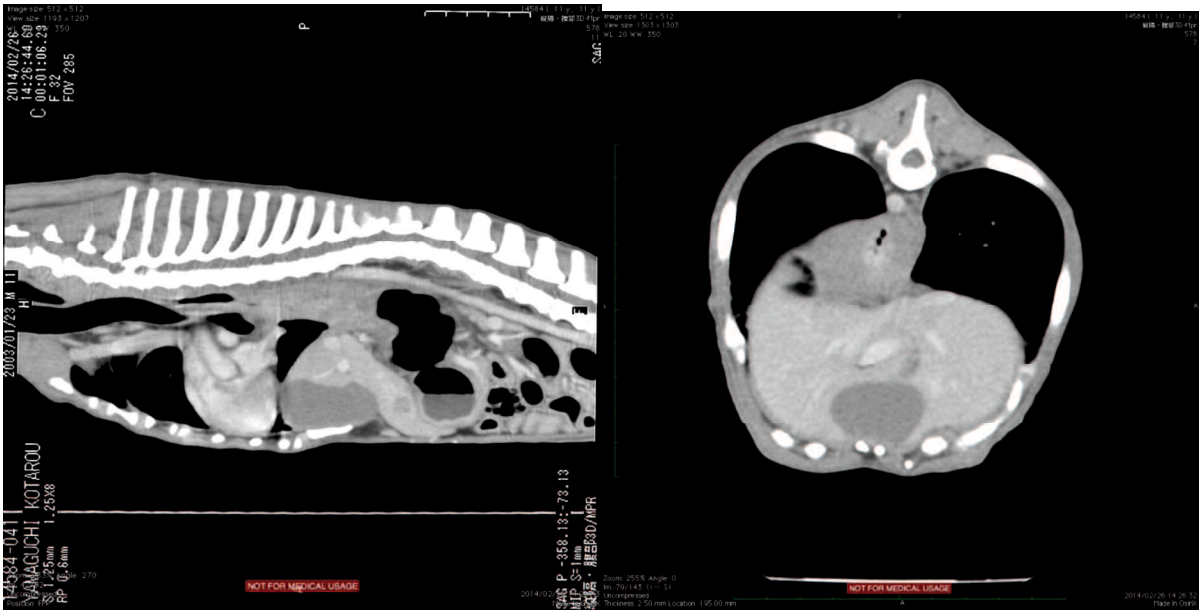


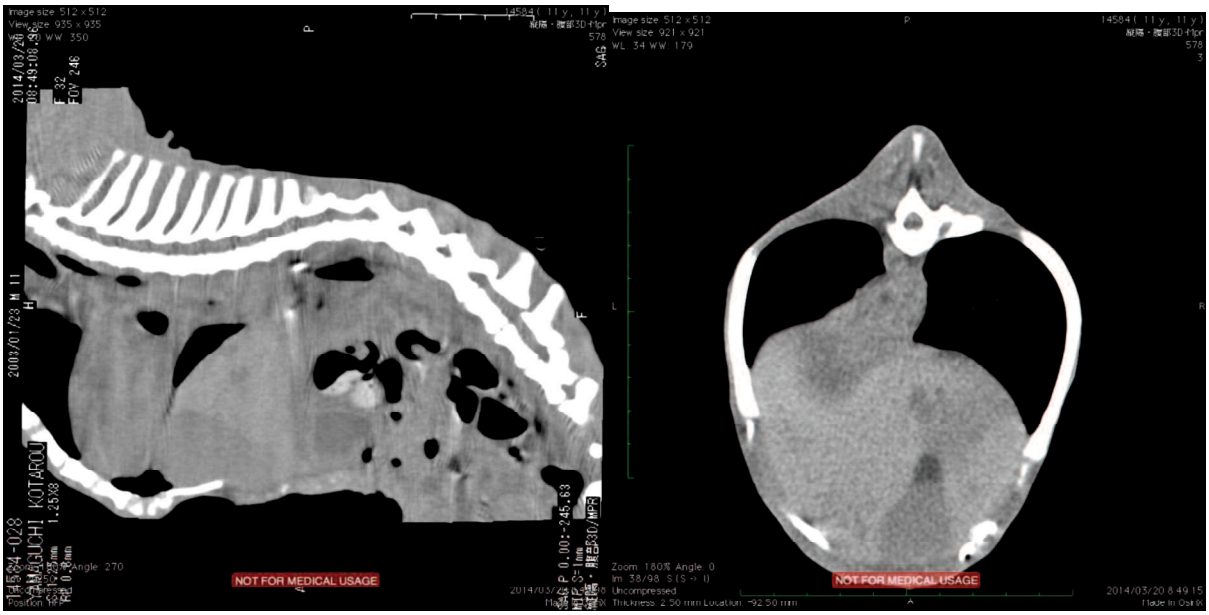
Fig. V-8: Patient 3 reovirus treatment cycle 2. (A) Pictures of mammary mass at pre-treatment of cycle 2 (cycle 1 day 23 post-treatment) and cycle 2 day 7 post-treatment. (B) Graph representing measurement of mammary mass during cycle 2 of reovirus treatment.

Figure 9

(A)



Pre-treatment

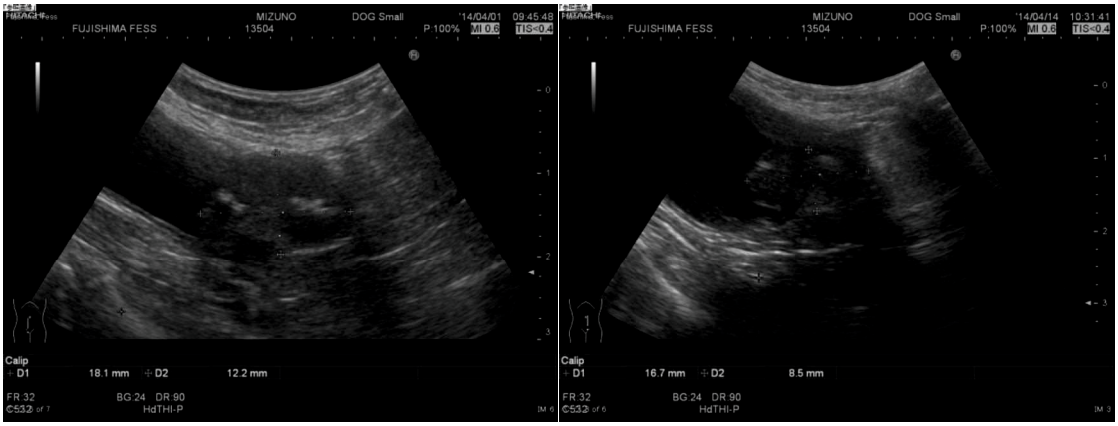


Day 7

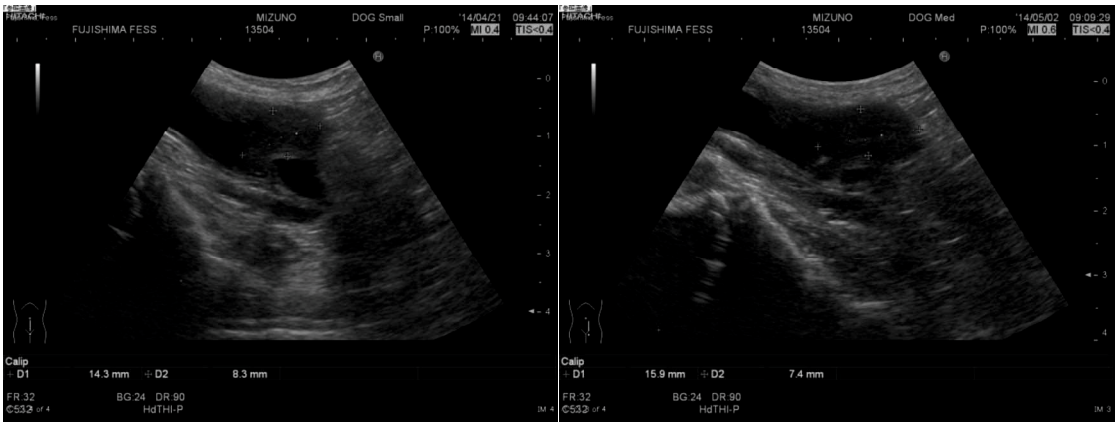
Fig. V-9: Patient 4. (A) CT images of esophageal mass pre-treatment and day 7 post-treatment.

Figure 10

(A)

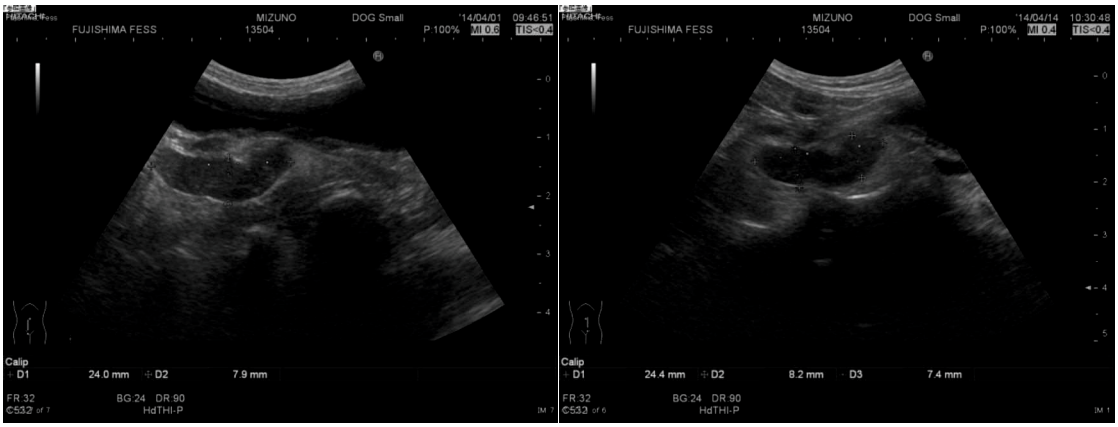


Pre-treatment　　Cycle 1 Day 13

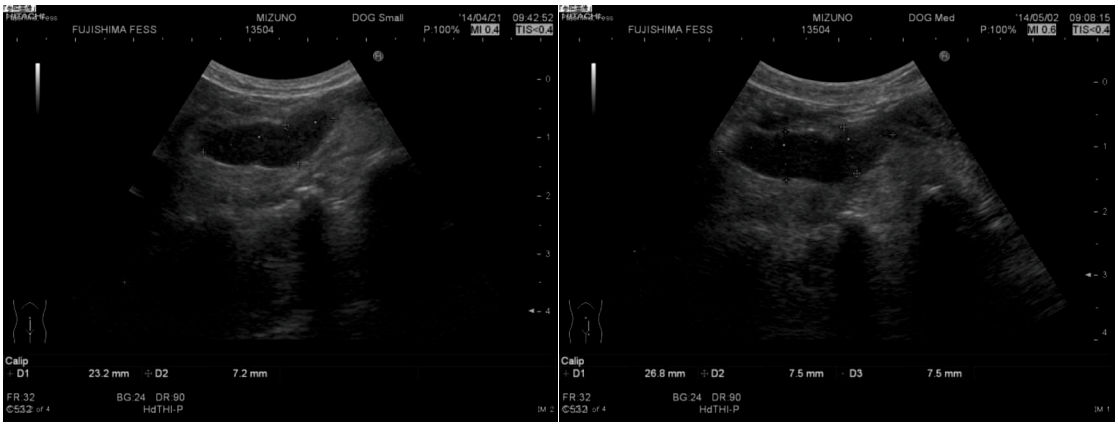


Cycle 2 Day 7　　Cycle 3 Day 11

(B)



Pre-treatment　　Cycle 1 Day 13



Cycle 2 Day 7 Cycle 3 Day 11

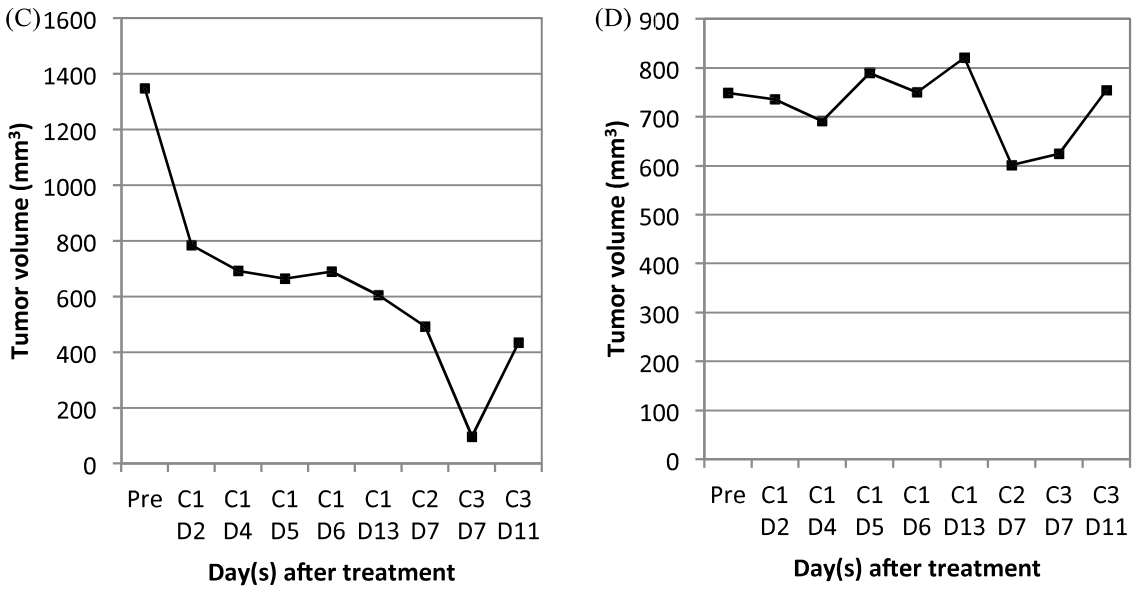


Fig. V-10: Patient 5. A) Ultrasound images of TCC mass pre-treatment, D13 post treatment cycle 1, D7 post treatment cycle 2 and D11 post treatment cycle 3. B) Ultrasound images of int./ ext. iliac lymph node during cycle 1, 2 and 3 of reovirus treatment. C) Graph representing size of TCC mass during cycle 1, 2 and 3 of reovirus treatment. D) Graph representing size of int./ ext. iliac lymph node during cycle 1, 2 and 3 of reovirus treatment.

CONCLUSION

For more than a century, viruses have been believed to be agents of cancer destruction. Interest in this field has been like a rollercoaster ride, where immense studies was conducted in the 1950s and 1960s, followed by a sudden neglect in the 1970s and 1980s before the next big wave of interest swept through these last three decades (Kelly and Russell, 2007). This resurgence of interest has thence allowed the rapid development a few oncolytic viruses up to phase III clinical trials in human cancers and the first oncolytic virus, adenovirus H101, was approved for marketing in China in 2005 (Garber, 2006). These milestones have certainly highlighted the potential of viruses, both naturally occurring and genetically modified, as one of the ultimate destroyers of cancers.

Even though the use of viruses as cancer therapy sounds promising, it is important that we do not forget the risk involved. One possible risk is the mutation of oncolytic virus that might lead to a trans-species transmission and infection. The feline panleukopenia virus, a parvovirus which used to be exclusive to the feline population, was able to evolve independently to be transmissible to dogs, resulting in the pandemic canine parvovirus that has infected more that 80% of wild and domestic dogs between 1978 and 1979 across the world (Parrish and Kawaoka, 2005). Even though the reason for this trans-species transmission is unknown, it might be noteworthy that this DNA virus has been tested as an oncolytic virus in humans before (Bierman et al., 1953). Another example comes from the Newcastle disease virus, an avian paramyxovirus, which is currently being administered in human clinical trials as an oncolytic virus (Russell et al, 2014). Despite the lack of adverse consequences, the development of this foreign virus as oncolytic virus for humans remains questionable. The introduction of wild-type viruses in a traditionally naïve host where the host populations have not evolved any resistance to the virus would be considered risky. The pathogen might acquire adaptations that could increase its virulence in a host not normally susceptible. This emphasized that the usage of virus as a cancer therapeutic agent should be

approached with caution after considerations from various perspectives.

Under the right circumstances, reovirus is capable of destroying tumor tissue via direct lysis. However, this direct oncolysis of reovirus is surrounded by multiple factors that hold stimulatory and inhibitory effects. Major barriers still limit the efficacy of these viruses, where the delicate fine-tuning of the balance between anti-viral and anti-tumor immunity is essential. Reports have indicated that the combination of reovirus and a therapy that can suppress the anti-viral immunity such as cyclophosphamide (Qiao et al., 2008) and radiotherapy (Harrington et al., 2010), can improve the oncolytic effects of reovirus. At the same time, the anti-tumor immunity response also plays essential roles in the elimination of cancer cells and protect the host against subsequent tumor challenges (Gujar et al., 2010). A recent report have highlighted that gemcitabine enhances the efficacy of reovirus through anti-tumoral responses (Gujar et al., 2014).

Even though all three serotypes of reovirus possess oncolytic ability (Alloussi et al., 2011), only the potential of the serotype 3 Dearing strain in canine cancer was investigated in my studies. In Chapter 1, canine mast cells of various origins, including canine mast cell tumor (MCT) cell lines, primary MCT cells and normal mast cells, were found to be susceptible to reovirus. Infection of these cells with reovirus was followed by a high production of viral progeny and apoptosis. The cytotoxic effects of reovirus were confirmed in MCT xenograft mouse models transplanted with a single intratumoral reovirus injection that induced marked tumor regression. However, the susceptibility of the cell lines towards reovirus did not have any correlation to the Ras activation status.

The following chapter focused on the screening of a panel of canine lymphoma cell lines infected with reovirus to assess the effects of reovirus in other canine cancers besides canine MCT. The susceptibility of canine lymphoma was not as high as expected, where only four out of ten cell lines underwent apoptosis and allowed replication of viral progeny after

reovirus infection. Similar to the canine MCT cell lines, there was no correlation between the Ras activation status and reoviral susceptibility among the canine lymphoma cell lines. Up to date, a total of nine canine mammary gland tumor, six melanoma and five osteosarcoma cell lines has been screen for their susceptibility towards reovirus in our laboratory and the results varied among the cell lines.

As there is inadequate information on the effects of reovirus in cell signaling pathways, we were determined to understand how reovirus utilizes different signaling pathways to exert its cytotoxic effects. Therefore, in Chapter 3, we showed that reovirus infection dysregulates the Ras/Raf/MEK/ERK pathway and degrades c-Kit as a common mean to induce apoptotic cell death. However, the difference of the susceptibility towards reovirus relies on its ability the dysregulation of the PI3K/Akt pathway. At this point, only these two major pathways were focused in our study. As cell signaling pathways are often complex, we were unable to rule out the involvement of other signaling pathways in reovirus oncolysis.

Chapter 4 highlighted the seroepidemiological studies of reovirus in six prefectures in Japan. The distribution of reovirus serotype 1, 2 and 3 is ubiquitous regardless of prefectures and area. Approximately 50% of the investigated canine population has antibodies against each of the reovirus serotypes due to natural infections. As concurrent infection of the three reovirus serotypes is possible, the existence of antibody against one serotype of reovirus does not automatically provide immunity against the other. This piece of information was invaluable in which the usage of the reovirus serotype 3 Dearing strain as an oncolytic therapy will not be hampered by the antibodies of other serotypes of reovirus.

Knowing that reovirus can induce tumor cell death in a range of canine cancers *in vitro*, clinical results involving reovirus were eagerly awaited. The final chapter is the most exciting chapter in my PhD thesis, which entails the works of reovirus in both clinical and laboratory settings. Results include data from the experimental infection of reovirus in healthy dogs,

followed by information of the preliminary study of reovirus in canine cancer patients. We concluded that the administration of 1.4 x 10^8 $TCID_{50}$ of reovirus in healthy dogs does not induce toxicities and viral shedding but stimulates a healthy neutralizing anti-reoviral antibody response. Among the five canine cancer patients, reovirus administration of a maximum of 5 x 10^9 $TCID_{50}$ for five consecutive days, either intratumorally or intravenously, was well-tolerated and adverse events were mild. Viral shedding was short-lived and all patients developed marked anti-viral immunity after reovirus therapy. This chapter has provided critical information on the actual usage of reovirus in a clinical setting, which will be used as a basis for the design and set up of a phase I clinical trial in the future.

Over the span of four years during my PhD studies, I have managed to experience research from bench to bedside, starting from the first sight of reovirus-induced cell death in canine cancer cell lines until the administration of reovirus in canine cancer patients. Based on all the results from Chapter 1 to 5, I was able to achieve most of the objectives for my PhD studies. That being said, there is still more work to be done to establish reovirus as an oncolytic virus for clinical usage. As compared to the studies of reovirus in human cancers, our current study is insufficient to provide evidence that reovirus is susceptible in a wide range of canine cancer. A general screening of a large panel of cancer cell lines of different origins is necessary in order to gather a census of cancer types that are susceptible to reovirus in dogs.

During my PhD studies, I was only able to investigate the effects of reovirus in two signaling pathways that has been reported most frequently. More time and effort are still needed to investigate the effects of reovirus in cells silenced or transformed with genes related to the Ras upstream effectors and the PI3K/Akt pathway. Therefore, I was unable to focus on the possible involvement of other pathways and provide a concrete conclusion whether the same pathways govern the susceptibility of reovirus between human and canine

cancers.

The administration reovirus has been proven to be safe in healthy dogs as the harmful effects of reovirus are attenuated by the neutralizing anti-viral antibodies. The five canine cancer patients involved in the preliminary study has allowed us to assess and understand the effects of reovirus in a clinical setting. However, a full fledge clinical trial for a new therapeutic agent requires the resources that are perhaps, unavailable in our current setting. However, with all these data, we are confident that we are able to design a phase I clinical trial for the determination of the maximal tolerable dose of reovirus and the assessment of toxicities in a larger group of canine cancer patient of a minimum of 20. Unfortunately, this phase I clinical trial might have to be conducted with the collaboration of other institute to guarantee success.

In our studies, we only focused on the usage of reovirus, which has a wide range of mammalian host. Up till now, adenoviruses, canine distemper virus, vaccinia virus and Canary Pox virus, are the only other viruses tested in canine cancers (Patil et al., 2012). This indicates that the development of oncolytic virotherapy for canine cancer is still at its infancy and there is a long way to go before this therapy can be approved for clinical usage. The limitations of this pioneering approach, including the efficacy, safety and clinical application, is by far the biggest holdback for oncolytic virotherapy in veterinary oncology. Even though both human and canine cancers share a lot of similarities, the approach towards cancer treatment is often different due to the variation in setting and system. Nonetheless, the application of the full potential of reovirus in canine cancers is something that will be exciting and full of surprises.

ACKNOWLEDGEMENTS

First and foremost, I'd like to thank GOD for all His strength and guidance throughout these four years in my PhD studies. His mercy and love has been unfailing in times of struggles and hardship.

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REFERENCES

Adair, R.A., Scott, K.J., Fraser, S., Errington-Mais, F., Pandha, H., Coffey, M., Selby, P., Cook, G.P., Vile, R., Harrington, K.J., Toogood, G., Melcher, A.A., 2012. Cytotoxic and immune-mediated killing of human colorectal cancer by reovirus-loaded blood and liver mononuclear cells. Int J Cancer 132:2327-2338.

Agazie, Y.M., Hayman, M.J., 2003. Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling. Mol Cell Biol 23:7875-7886.

Alain, T., Hirasawa, K., Pon, K.J., Nishikawa, S.G., Urbanski, S.J., Auer, Y., Luider, J., Martin, A., Johnston, R.N., Janowska-Wieczorek, A., Lee, P.W., Kossakowska, A.E., 2002. Reovirus therapy of lymphoid malignancies. Blood 100:4146-4153.

Alain, T., Kim, M., Johnston, R.N., Urbanski, S., Kossakowska, A.E., Forsyth, P.A., Lee, P.W.K., 2006. The oncolytic effect in vivo of reovirus on tumour cells that have survived reovirus cell killing in vitro. Brit J Cancer 95, 1020-1027.

Alain, T., Kim, T.S., Lun, X., Liacini, A., Schiff, L.A., Senger, D.L., Forsyth, P.A., 2007. Proteolytic disassembly is a critical determinant for reovirus oncolysis. Mol Ther 15:1512-1521.

Alain, T., Muzik, H., Otsuka, S., Chan, S., Magliocco, T., Diaz, R., Forsyth, P.A., Morris, D., Bebb, G., 2010. Susceptibility of mantle cell lymphomas to reovirus oncolysis. Leukemia Res 34:100-108.

Alloussi, S.H., Alkassar, M., Urbschat, S., Graf, N., Gärtner, B., 2011. All reovirus subtypes show oncolytic potential in primary cells of human high-grade glioma. Oncol Rep 26:645-649.

Aoki, M., Batista, O., Bellacosa, A., Tsichlis, P., Vogt, P.K., 1998. The Akt kinase: molecular determinants of oncogenicity. Proc Natl Acad Sci USA 95:14950-14955.

Barton, E.S., Connolly, J.L., Forrest, J.C., Chappell, J.D., Dermody, T.S., 2001. Utilization of sialic acid as a coreceptor enhances reovirus attachment by multistep adhesion strengthening. J Biol Chem 276:2200-2211.

Barton, E.S., Forrest, J.C., Connolly, J.L., Chappell, J.D., Liu, Y., Schnell, F.J., Nusrat, A., Parkos, C.A., Dermody, T.S., 2001. Junction adhesion molecule is a receptor for reovirus. Cell 104:441-451.

Begg, C., Cho, M., Eastwood, S., Horton, R., Moher, D., Olkin, I., Pitkin, R., Rennie, D., Schulz, K.F., Simel, D., Stroup, D.F., 1996. Improving the quality of reporting of randomized controlled trials. The CONSORT statement. JAMA 276:637-639.

Berger, A.K., Danthi, P., 2013. Reovirus activates a caspase-independent cell death pathway. mBio 4:e00178-13.

Berard, A., Coombs, K.M., 2009. Mammalian reoviruses: propagation, quantification, and storage. Curr Protoc Microbiol Chapter 15:Unit15C.1.

Bierman, H.R., Crile, D.M., Dod, K.S., Kelly, K.H., Petrakis, N.L., White, L.P., Shimkin, M.B., 1953. Remissions in leukemia of childhood following acute infectious disease: staphylococcus and streptococcus, varicella, and feline panleukopenia. Cancer 6:591-605.

Binn, L.N., Marchwicki, R.H., Keenan, K.P., Strano, A.J., Engler, W.F., 1977. Recovery of reovirus type 2 from an immature dog with respiratory tract disease. Am J Vet Res 38:927-929.

Bos, J.L., 1989. ras oncogenes in human cancer: a review. Cancer Res 49:4682-4689.

Buonavoglia, C., Martella, V., 2007. Canine respiratory viruses. Vet Res 38:355-373.

Butler, L.M., Bonnett, B.N., Page, R.L., 2013. Epidemiology and the evidence-based medicine approach. In: Small animal clinical oncology, Fifth Ed., edited by Withrow, S.J., Vail, D.M., Page, R.L., Saunders Elsevier, St. Louis, MO, USA, pp. 68-82.

Cagnol, S., Chambard, J.C., 2010. ERK and cell death: mechanisms of ERK-induced cell death- apoptosis, autophagy and senescence. EBS J. 277:2-21.

Chan, E.Y.W., Stang, S.L., Bottorff, D.A., Stone, J.C., Stone, 1999. Hypothermic stress leads to activation of Ras-Erk signaling. J Clin Invest 103:1337-1344.

Chappell, J.D., Gunn, V.L., Wetzel, J.D., Baer, G.S., Dermody, T.S., 1997. Mutations in type 3 reovirus that determine binding to sialic acid are contained in the fibrous tail domain of viral attachment protein sigma1. J Virol 71:1834-1841.

Cho, I.R., Koh, S.S., Min, H.J., Park, E.H., Srisuttee, R., Jhun, B.H., Kang, C.D., Kim, M., Johnston, R.N., Chung, Y.H., 2010. Reovirus infection induces apoptosis of TRAIL-resistant gastric cancer cells by down-regulation of Akt activation. Int J Oncol 36:1023-1030.

Clarke, P., Meintzer, S.M., Widmann, C., Johnson, G.L., Tyler, K.L., 2001. Reovirus infection activates JNK and the JNK-dependent transcription factor c-Jun. J Virol 75:11275-11283.

Clarke, P., Meintzer, S.M., Wang, Y., Moffitt, L.A., Richardson-Burns, S.M., Johnson, G.L., Tyler, K.L., 2004. JNK regulates the release of proapoptotic mitochondrial factors in reovirus-infected cells. J Virol 78:13132-13138.

Coffey, M.C., Strong, J.E., Forsyth, P.A., Lee, P.W., 1998. Reovirus therapy of tumors with activated Ras pathway. Science 282:1332-1334.

Comins, C., Spicer, J., Protheroe, A., Roulstone, V., Twigger, K., White, C.M., Vile, R., Melcher, A., Coffey, M.C., Mettinger, K.L., Nuovo, G., Cohn, D.E., Phelps, M., Harrington, K.J., Pandha, H.S., 2010. Reo-10: A Phase I study of intravenous reovirus and docetaxel in patients with advanced cancer. Clin Cancer Res 16:5564–5572.

Connolly, J.L., Rodgers, S.E., Clarke, P., Ballard, D.W., Kerr, L.D., Tyler, K.L., Dermody, T.S., 2000. Reovirus-induced apoptosis requires activation of transcription factor NF-κB. J Virol 74:2981-2989.

Csiza, C. K. 1974. Characterization and serotyping of three feline reovirus isolates. Infect Immun 9:159-166.

Dance, M., Montagner, A., Salles, J.P., Yart, A., Raynal, P., 2008. The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway. Cell Signal 20:453-459.

Dankort, D., Maslikowski, B., Warner, N., Kanno, N., Kim, H., Wang, Z., Moran, M.F., Oshima, R.G., Cardiff, R.D., Muller, W.J., 2001. Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: implications for human breast cancer. Mol Cell Biol 21:1540-1551.

Davis, J.J., Fang, B., 2005. Oncolytic virotherapy for cancer treatment: challenges and solutions. J Gene Med 7:1380-1389.

Decaro, N., Campolo, M., Desario, C., Ricci, D., Camero, M., Lorusso, E., Elia, G., Lavazza, A., Martella, V., Buonavoglia, C., 2005. Virological and molecular characterization of a mammalian orthoreovirus type 3 strain isolated from a dog in Italy. Vet Microbiol 109:19-27.

Dock, G., 1904. The influence of complicating diseases upon leukemia. Am J Med Sci 127:563-592.

Duncan, M.R., Stanish, S.M., Cox, D.C., 1978. Differential sensitivity of normal and transformed human cells to reovirus infection. J Virol 28:444-449.

Errington, F., Steele, L., Prestwich, R., Harrington, K.J., Pandha, H.S., Vidal, L., de Bono, J., Selby, P., Coffey, M., Vile, R., Melcher, A., 2008. Reovirus activates human dendritic cells to promote innate antitumor immunity. J Immunol 180:6018-6026.

Errington, F., White, C.L., Twigger, K.R., Rose, A., Scott, K., Steele, L., Ilett, L.J., Prestwich, R., Pandha, H.S., Coffey, M., Selby, P., Vile, R., Harrington, K.J., Melcher, A.A., 2008. Inflammatory tumour cell killing by oncolytic reovirus for the treatment of melanoma. Gene Ther 15:1257-1270.

Escobar, H.M., Gunther, K., Ritcher, A., Soller, J.T., Winkler, S., Nolte, I., Bulerdiek, J., 2004. Absence of ras-gene hot-spot mutations in canine fibrosarcomas and melanomas. Anticancer Res 24:3027-3028.

Etoh, T., Himeno, Y., Matsumoto, T., Aramaki, M., Kawano, K., Nishizono, A., Kitano, S., 2003. Oncolytic viral therapy for human pancreatic cancer cells by reovirus. Clin Cancer Res 9:1218-1223.

Fukumi, H., Takeuchi, Y., Ishida, M., Saito, H., 1969. Serological epidemiology of reovirus infection. 1. Jpn J Med Sci Biol 22:13-21.

Forsyth, P., Roldán, G., George, D., Wallace, C., Palmer, C.A., Morris, D., Cairncross, G., Matthews, M.V., Markert, J., Gillespie, Y., Coffey, M., Thompson, B., Hamilton, M., 2008. A Phase I Trial of Intratumoral Administration of Reovirus in Patients with Histologically Confirmed Recurrent Malignant Gliomas. Mol Ther 16:627–632.

Fournel-Fleury, C., Magnol, J.P., Bricaire, P., Marchal, T., Chabanne, L., Delverdier, A., Bryon, P.A., Felman, P., 1997. Cytohistological and immunological classification of canine malignant lymphomas: comparison with human non-Hodgkin's lymphomas. J Comp Pathol 117:35-59.

Galanis, E., Markovic, S.N., Suman, V., Nuovo, G., Vile, R., Coffey, M., Linette, G., Maples, W., Zwiebel, J., Kendra, K., 2011. Phase II trial of intravenous administration of Reolysin (Reovirus Serotype-3-Dearing Strain) in patients with metastatic melanoma. Mol Ther 19:1375.

Garber, K., 2006. China approves world's first oncolytic virus therapy for cancer treatment. J Natl Cancer Inst 98:298-300.

Georgopoulou, U., Caravokiri, K., Mavromara, P., 2003. Suppression of the ERK1/2 signaling pathway from HCV NS5A protein expressed by herpes simplex recombinant viruses. Arch Virol 148:237-251.

Gollamudi, R., Ghalib, M.H., Desai, K.K., Chaudhary, I., Wong, B., Einstein, M., Coffey, M., Gill, G.M., Mettinger, K., Mariadason, J.M., Mani, S., Goel, S., 2010. Intravenous administration of Reolysin®, a live replication competent RNA virus is safe in patients with advanced solid tumors. Invest New Drugs 28:641–649.

Gordon, I.K., Khanna, C., 2010. Modeling opportunites in comparative oncology for drug development. ILAR J 51:214-220.

Griffey, S.M., Kraegel, S.A., Madewell, B.R., 1998. Rapid detection of K-ras gene mutations in canine lung cancer using single-strand conformational polymorphism analysis. Carcinogenesis 19:959-963.

Gujar, S.A., Clements, D., Dielschneider, R., Helson, E., Marcato, P., Lee, P.W.K., 2014. Gemcitabine enhances the efficacy of reovirus-based oncotherapy through anti-tumour immunological mechanisms. Br J Cancer 110:83-93.

Gujar, S.A., Marcato, P., Pan, D., Lee, P.W., 2010. Reovirus virotherapy overrides tumor antigen presentation evasion and promotes protective antitumor immunity. Mol Cancer Ther 9:2924-2933.

Harrington, K.J., Karapanagiotou, E.M., Roulstone, V., Twigger, K.R., White, C.L., Vidal, L., Beirne, D., Prestwich, R., Newbold, K., Ahmed, M., Thway, K., Nutting, C.M., Coffey, M., Harris, D., Vile, R.G., Pandha, H.S., De bono, J.S., Melcher, A.A., 2010. Two-stage phase I dose-escalation study of intratumoral reovirus type 3 dearing and palliative radiotherapy in patients with advanced cancers. Clin Cancer Res 16:3067-3077.

Hashiro, G., Loh, P.C., Yau, J.T., 1977. The preferential cytotoxicity of reovirus for certain transformed cell lines. Arch Virol 54:307-315.

Hata, Y., Etoh, T., Inomata, M., Shiraishi, N., Nishizono, A., Kitano, S., 2008. Efficacy of oncolytic reovirus against human breast cancer cells. Oncol Rep 19:1395-1398.

Heinrich, M.C., Blanke, C.D., Druker, B.J., Corless, C.L., 2002. Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies. J Clin Oncol 20:1692-1703.

Hirahara, T., Yasuhara, H., Matsui, O., Kodama, K., Nakai, M., Sasaki, N., 1988. Characteristics of reovirus type 1 from the respiratory tract of pigs in Japan. Jpn J Vet Sci 50:353-361.

Hirasawa, K., Nishikawa, S.G., Norman, K.L., Alain, T., Kossakowska, A., Lee, P.W.K., 2002. Oncolytic reovirus against ovarian and colon cancer. Cancer Res 62:1696-1701.

Hirasawa, K., Nishikawa, S.G., Norman, K.L., Coffey, M.C., Thompson, B.G., Yoon, C.S., Waisman, D.M., Lee, P.W., 2003. Systemic reovirus therapy of metastatic cancer in immune-competent mice. Cancer Res 63:348-353.

Hrdy, D.B., Rosen, L., Fields, B.N., 1979. Polymorphism of the migration of double-stranded RNA genome segments of reovirus isolates from humans, cattle, and mice. J Virol 31:104-111.

Ikeda, Y., Nishimura, G., Yanoma, S., Kubota, A., Furukawa, M., Tsukuda, M., 2004. Reovirus oncolysis in human head and neck squamous carcinoma cells. Auris Nasus Larynx 31:407-412.

Ilett, E.J., Bárcena, M., Errington-Mais, F., Griffin, S., Harrington, K.J., Pandha, H.S., Coffey, M., Selby, P.J., Limpens, R.W., Mommaas, M., Hoeben, R.C., Vile, R.G., Melcher, A.A., 2011. Internalization of oncolytic reovirus by human dendritic cell carriers protects the virus from neutralization. Clin Cancer Res 17:2767-2776.

Ishiguro, T., Kadosawa, T., Mori, K., Takagi, S., Okumura, M., Fujinaga, T., 2001. Establishment and characterization of a new canine mast cell tumor cell line. J Vet Med Sci 63:1031-1034.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA Cancer J Clin 61:69-90.

Kalesnikoff, J., Galli, S.J., 2011. Antiinflammatory and immunosuppressive functions of mast cells. Methods Mol Biol 677:207-220.

Karapanagiotou, E.M., Roulstone, V., Twigger, K., Ball, M., Tanay, M., Nutting, C., Newbold, K., Gore, M.E., Larkin, J., Syrigos, K.N., Coffey, M., Thompson, B., Mettinger, K., Vile, R.G., Pandha, H.S., Hall, G.D., Melcher, A.A., Chester, J., Harrington, K.J., 2012. Phase I/II trial of carboplatin and paclitaxel chemotherapy in combination with intravenous oncolytic reovirus in patients with advanced malignancies. Clin Cancer Res 18:2080-2089.

Kawaguchi, K., Etoh, T., Suzuki, K., Mitui, M.T., Nishizono, A., Shiraishi, N., Kitano, S., 2010. Efficacy of oncolytic reovirus against human gastric cancer with peritoneal metastasis in experimental animal model. Int J Oncol 37:1433-1438.

Kawarai S, Masuda K, Ohmori K, Matsuura S, Yasuda N, Nagata, M., Sakaguchi, M., Tsujimoto, H., 2009. Cultivation and characterization of canine skin-derived mast cells. J Vet Med Sci 72:131-140.

Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordán, J., Bellacosa, A., Tsichlis, P.N., Hay, N., 1997. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev 11:701-713.

Kelly, E., Russell, S.J., 2007. History of Oncolytic Viruses: Genesis to Genetic Engineering. Mol Ther 15:651-659.

Kilani, R.T., Tamimi, Y., Hanel, E.G., Wong, K.K., Karmali, S., Lee, P.W.K., Moore, R.B., 2003. Selective reovirus killing of bladder cancer in a co-culture spheroid model. Virus Res 93:1-12.

Kim, M.J., Chang, J.S., Park, S.K., Hwang, J.I., Ryu, S.H., Suh, P.G., 2000. Direct interaction of SOS1 ras exchange protein with the SH3 domain of phospholipase C-γ1. Biochem 39:8674-8682.

Kim, M., Egan, C., Alain, T., Urbanski, S.J., Lee, P.W., Forsyth, P.A., Johnston, R.N., 2007. Acquired resistance to reoviral oncolysis in Ras-transformed fibrosarcoma cells. Oncogene 26:4124-4134.

Kim, M., Williamson, C.T., Prudhomme, J., Bebb, D.G., Riabowol, K., Lee, P.W., Lees-Miller, S.P., Mori, Y., Rahman, M.M., McFadden, G., Johnston, R.N., 2010. The viral tropism of two distinct oncolytic viruses, reovirus and myxoma virus, is modulated by cellular tumor suppressor gene status. Oncogene 29:3990-3996.

Kokubu, T., Takahashi, T., Takamura, K., Yasuda, H., Hiramatsu, K., Nakai, M., 1993. Isolation of reovirus type 3 from dogs with diarrhea. J Vet Med Sci 55:453-454.

Lerner, A.M., Cherry, J.D., Klein, J.O., Finland, M., 1962. Infections with reoviruses. N Engl J Med 267:947-952.

Levine, R.A., Forest, T., Smith, C., 2002. Tumor suppressor PTEN is mutated in canine osteosarcoma cell lines and tumors. Vet Pathol 39:372-378.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H., Parsons, R., 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943-1947.

Liang, J., Wu, Y.L., Chen, B.J., Zhang, W., Tanaka, Y., Sugiyama, H., 2013. The c-Kit receptor-mediated signal transduction and tumor-related diseases. Int J Biol Sci 9:435-443.

Lin, T.Y., Rush, L.J., London, C.A., 2006. Generation and characterization of bone marrow-derived cultured canine mast cells. Vet Immunol Immunopathol 113:37-52.

Linnekin, D., 1999. Early signaling pathways activated by c-Kit in hematopoietic cells. Int J Biochem Cell Biol 31:1053-1074.

Loken, S.D., Norman, K., Hirasawa, K., Nodwell, M., Lester, W.M., Demetrick, D.J., 2004. Morbidity in immunosuppressed (SCID/NOD) mice treated with reovirus (Dearing 3) as an anti-cancer biotherapeutic. Cancer Biol Ther 3:734-738.

Lolkema, M.P., Arkenau, H.T., Harrington, K., Roxburgh, P., Morrison, R., Roulstone, V., Twigger, K., Coffey, M., Mettinger, K., Gill, G., Evans, T.R.J., de Bono, J.S., 2011. A phase I study of the combination of intravenous reovirus type 3 Dearing and gemcitabine in patients with advanced cancer. Clin Cancer Res 17:581-588.

London, C.A., Thamm, D.H., 2013. Mast cell tumors. In: Small animal clinical oncology, Fifth Ed., edited by Withrow, S.J., Vail, D.M., Page, R.L., Saunders Elsevier, St. Louis, MO, USA, pp. 335-355.

Lou, T.Y., Wenner, H.A., 1963. Natural and experimental infection of dogs with reovirus type 1: pathogenecity of the strain for other animals. Am J Hyg 77:293-304.

Macdonald, A., Crowder, K., Street, A., McCormick, C., Saksela, K., Harris, M., 2003. The hepatitis C virus non-structural NS5A protein inhibits activating protein-1 function by perturbing ras-ERK pathway signaling. J Biol Chem 278:17775-17784.

Maitra, R., Ghalib, M.H., Goel, S., 2012. Reovirus: a targeted therapeutic- progress and potential. Mol Cancer Res 10:1514-1525.

Marcato, P., Shmulevitz, M., Pan, D., Stoltz, D., Lee, P.W., 2007. Ras transformation mediates reovirus oncolysis by enhancing virus uncoating, particle infectivity, and apoptosis-dependent release. Mol Ther 15:1522-1530.

Marconato, L., 2011. The staging and treatment of multicentric high-grade lymphoma in dogs: A review of recent developments and future prospects. Vet J 188:34-38.

Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D.R., Zilberstein, A., Ullrich, A., Pawson, T., Schlessinger, J., 1990. The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. EMBO J 9:4375-4380.

Marshall, J.A., Kennett, M.L., Rodger, S.M., Studdert, M.J., Thompson, W.L., Gust, I.D., 1987. Virus and virus-like particles in the faeces of cats with and without diarrhoea. Aust Vet J 64:100-105.

Massie, E.L., Shaw, E.D., 1966. Reovirus type 1 in laboratory dogs. Am J Vet Res 27:783-787.

Matsuura, K., Hasegawa, S., Nakayama, T., Morita, O., Uetake, H., 1984. Viral pollution of the rivers in Toyama City. Microbiol Immunol 28:575-588.

Mayr, B., Winkler, G., Schaffner, G., Reifinger, M., Brem, G., 2002. N-ras mutation in a feline lymphoma. Low frequency of N-ras mutations in a series of feline, canine and bovine lymphomas. Vet J 163:326-328.

McCubrey, J.A., Steelman, L.S., Abrams, S.L., Lee, J.T., Chang, F., Bertrand, F.E., Navolanic, P.M., Terrian, D.M., Franklin, R.A., D'Assoro, A.B., Salisbury, J.L., Mazzarino, M.C., Stivala, F., Libra, M., 2006. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Advan Enzyme Regul 46:249-279.

Meerani, S., Yao, Y., 2010. Oncolytic viruses in cancer therapy. Eur J Sci Res 40:156-171.

Metcalfe, D.D., 2005. Regulation of normal and neoplastic human mast cell development in mastocytosis. Trans Am Clin Climatol Assoc 116:185-205.

Miao, J., Chen, G.A., Yun, J.P., Chun, S.Y., Zheng, Z.Z., Ho, R.L.K., Chak, E.C.W., Xia, N.S., Lai, P.B.S., 2007. Identification and characterization of BH3 domain protein Bim and its isoforms in human hepatocellular carcinomas. Apoptosis 12:1691-1701.

Mochizuki, M., Uchizono, S., 1993. Experimental infections of feline reovirus serotype 2 isolates. J Vet Med Sci 55:469-470.

Momoi, Y., Okai, Y., Watari, T., Goitsuka, R., Tsujimoto, H., Hasegawa, A., 1997. Establishment and characterization of a canine T-lymphoblastoid cell line derived from malignant lymphoma. Vet Immunol Immunopathol 59:11-20.

Morris, D.G., Feng, X., DiFrancesco, L.M., Fonseca, K., Forsyth, P.A., Paterson, A.H., Coffey, M.C., Thompson, B., 2013. REO-001: A phase I trial of percutaneous intralesional administration of reovirus type 3 dearing (Reolysin®) in patients with advanced solid tumors. Invest New Drugs 31:696–706.

Murakami, T., Kato, H., 1975. Reovirus antibody patterns in dogs: a trial for the application of principal component analysis to seroepidemiology. Natl Inst Anim Health Q (Tokyo) 15:8-14.

Murua Escobar, H., Günther, K., Richter, A., Soller, J.T., Winkler, S., Nolte, I., Bullerdiek, J., 2004. Absence of ras-gene hot-spot mutations in canine fibrosarcomas and melanomas. Anticancer Res 24:3027-3028.

Muscillo, M., La Rosa, G., Marianelli, C., Zaniratti, S., Capobianchi, M. R., Cantiani, L., Carducci, A. 2001. A new RT-PCR method for the identification of reoviruses in seawater samples. Water Res 35:548-556.

Nakaichi, M., Taura, Y., Kanki, M., Mamba, K., Momoi, Y., Tsujimoto, H., Nakama, S., 1996. Establishment and characterization of a new canine B-cell leukemia cell line. J Vet Med Sci 58:469-471.

NCI, 2013 http://www.cancer.gov/cancertopics/factsheet/clinicaltrials/clinical-trials

Nguyen, S.M., Thamm, D.H., Vail, D.M., London, C.A., 2013. Response evaluation criteria for solid tumours in dogs (v1.0): a Veterinary Cooperative Oncology Group (VCOG) consensus document. Vet Comp Oncol [Epub ahead of print]

Norman, K.L., Coffey, M.C., Hirasawa, K., Demetrick, D.J., Nishikawa, S.G., DiFrancesco, L.M., Strong, J.E., Lee, P.W.K., 2002. Reovirus oncolysis of human breast cancer. Human Gene Ther 13:641-652.

Norman, K.L., Hirasawa, K., Yang, A.D., Shields, M.A., Lee, P.W., 2004. Reovirus oncolysis: the Ras/RalGEF/p38 pathway dictates host cell permissiveness to reovirus infection. Proc Natl Acad Sci USA 101:11099-11104.

Oh-hora, M., Johmura, S., Hashimoto, A., Hikida, M., Kurosaki, T., 2003. Requirement for ras guanine nucleotide releasing protein 3 in coupling phospholipase C- γ2 to ras in B cell receptor signaling. J Exp Med 198:1841-1851.

Ohmori, K., Kawarai, S., Yasuda, N., Tanaka, A., Matsuda, H., Nishimura, R., Sasaki, N., Tsujimoto, H., Masuda, K., 2008. Identification of c-kit mutations-independent neoplastic cell proliferation of canine mast cells. Vet Immunol Immunopathol 126:43-53.

Olayioye, M.A., Neve, R.M., Lane, H.A., Hynes, N.E., 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 19:3159-3167.

Osterhaus, A., Berghuis-De Vries, J., Steur, K., 1977. Antiviral antibodies in dogs in the Netherlands. Zentralbl Veterinarmed B 24:123-133.

Ouattara, L.A., Barin, F., Barthez, M.A., Bonnaud, B., Roingeard, P., Goudeau, A., Castelnau, P., Vernet, G., Paranhos-Baccalà, G., Komurian-Pradel, F., 2011. Novel human reovirus isolated from children with acute necrotizing encephalopathy. Emerg Infect Dis 17:1436-1444.

Pal, S.R., Agarwal, S.C., 1968. Sero-epidemiological study of reovirus infection amongst the normal population of the Chandigarh area- northern India. J Hyg (Lond) 66:519-529.

Pan, D., Marcato, P., Ahn, D.G., Gujar, S., Pan, L.Z., Shmulevitz, M., Lee, P.W., 2013. Activation of p53 by chemotherapeutic agents enhances reovirus oncolysis. PLoS ONE 8: e54006.

Paoloni, M., Khanna, C., 2007. Comparative oncology today. Vet Clin North Am Small Anim Pract 37:1023-1032.

Paoloni, M., Khanna, C., 2008. Translation of new cancer treatments from pet dogs to humans. Nat Rev Cancer 8:147-156.

Parrish, C.R., Kawaoka, Y., 2005. The origins of new pandemic viruses: the acquisition of new host ranges by canine parvovirus and influenza A viruses. Annu Rev Microbiol 59:553-586.

Patil, S.S., Gentschev, I., Nolte, I., Ogilvie, G., Szalay, A.A., 2012. Oncolytic virotherapy in veterinary medicine: current status and future prospects for canine patients. J Transl Med 10:3.

Peyssonnaux, C., Provot, S., Felder-Schmittbuhl, M.P., Calothy, G., Eychène, A., 2000. Induction of postmitotic neuroretina cell proliferation by distinct Ras downstream signaling pathways. Mol Cell Biol 20:7068-7079.

Prestwich, R.J., Errington, F., Diaz, R.M., Pandha, H.S., Harrington, K.J., Melcher, A.A., Vile, R.G., 2009. The case of oncolytic viruses versus the immune system: waiting on the judgment of Solomon. Hum Gene Ther 20:1119-1132.

Qiao, J., Wang, H., Kottke, T., White, C., Twigger, K., Diaz, R.M., Thompson, J., Selby, P., de Bono, J., Melcher, A., Pandha, H., Coffey, M., Vile, R., Harrington, K., 2008. Cyclophosphamide facilitates antitumor efficacy against subcutaneous tumors following intravenous delivery of reovirus. Clin Cancer Res 14:259-269.

Rahaus, M., Desloges, N., Wolff, M.H., 2006. Varicella-zoster virus influences the activities of components and targets of the ERK signaling pathway. J Gen Virol 87:749-758.

Ramos-Alvarez, M., Sabin, A.B., 1958. Enteropathogenic viruses and bacteria. Role in summer diarrheal diseases of infancy and early childhood. JAMA 167:147-156.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 27:493-497.

Rosen, L. 1960. Serologic grouping of reoviruses by hemagglutination-inhibition. Am J Hyg 71:242-249.

Rosen, L., Hovis, J.F., Mastrota, F.M., Bell, J.A., Huebner, R.J., 1960. Observations on a newly recognized virus (Abney) of the reovirus family. Am J Hyg 71:258-265.

Rosen, L. 1962. Reoviruses in animals other than man. Ann N Y Acad Sci 101:461-465.

Rosen, L., Abinanti, F.R., Hovis, J.F., 1963. Further observations on the natural infection of cattle with reoviruses. Am J Hyg 77:38-48.

Rosen, L., Evans, H.E., Spickard, A., 1963. Reovirus infections in human volunteers. Am J Hyg 77:29-37.

Roskoski, R., 2005. Structure and regulation of Kit protein-tyrosine kinase- the stem cell factor receptor. Biochem Biophys Res Commun 338:1307-1315.

Russell, S.J., Peng, K.W., Bell, J.C., 2012. Oncolytic virotherapy. Nat Biotechnol 30:658-670.

Rütgen, B.C., Hammer, S.E., Gerner, W., Christian, M., de Arespacochaga, A.G., Willmann, M., Kleiter, M., Schwendenwein, I., Saalmuller, A., 2010. Establishment and characterization of a novel canine B-cell line derived from a spontaneously occurring diffuse large cell lymphoma. Leukemia Res 34:932-938.

Sabin, A.B., 1959. Reoviruses. Science 130:1389-1397.

Sahin, E., Egger, M.E., McMasters, K.M., Zhou, H.S., 2013. Development of oncolytic reovirus for cancer therapy. J Cancer Ther 4:1100-1115.

Schiff, L.A., Nibert, M.L., Tyler, K.L., 2007. Orthoreoviruses and their replication. In: Fields Virology, Fifth Ed., edited by Knipe, D.M., Howley, P.M., Lippincott Williams & Wilkins, Philadelphia, PA, USA, pp. 1853-1915.

Schumann, M., Dobbelstein, M., 2006. Adenovirus-induced extracellular signal-regulated kinase phosphorylation during the late phase of infection enhances viral protein levels and virus progeny. Cancer Res 66:1282-1288.

Scott, F.W., Kahn, D.E., Gillespie, J.H., 1970. Feline viruses: isolation, characterization, and pathogenicity of a feline reovirus. Am J Vet Res 31:11-20.

Sei, S., Mussio, J.K., Yang, Q.E., Nagashima, K., Parchment, R.E., Coffey, M.C., Shoemaker, R.H., Tomaszewski, J.E., 2009. Synergistic antitumor activity of oncolytic reovirus and chemotherapeutic agents in non-small cell lung cancer cells. Mol Cancer 8:47.

Selb, B., Weber, B., 1994. A study of human reovirus IgG and IgA antibodies by ELISA and western blot. J Virol Methods 47:15-25.

Shmulevitz, M., Marcato, P., Lee, P.W., 2005. Unshackling the links between reovirus oncolysis, Ras signaling, translational control and cancer. Oncogene 24:7720-7728.

Smakman, N., van den Wollenberg, D.J., Elias, S.G., Sasazuki, T., Shirasawa, S., Hoeben, R.C., Borel Rinkes, I.H., Kranenburg, O., 2006. KRAS (D13) Promotes apoptosis of human colorectal tumor cells by ReovirusT3D and oxaliplatin but not by tumor necrosis factor-related apoptosis-inducing ligand. Cancer Res 66:5403-5408.

Song, L., Ohnuma, T., Gelman, I.H., Holland, J.F., 2009. Reovirus infection of cancer cells is not due to activated Ras pathway. Cancer Gene Ther 16:382.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H., Tavtigian, S.V., 1997. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 15:356-362.

Steelman, L.S., Chappell, W.H., Abrams, S.L., Kempf C.R., Long, J., Laidler, P., Mijatovic, S., Maksimovic-Ivanic, D., Stivala, F., Mazzarino, M.C., Donia, M., Fagone, P., Malaponte, G., Nicoletti, F., Libra, M., Milella, M., Tafuri, A., Bonati, A., Bäsecke, J., Cocco, L., Evangelisti, C., Martelli, A.M., Montalto, G., Cervello, M., McCubrey, J.A., 2011. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. Aging 3:192-222.

Steplewski, Z., Jeglum, K.A., Rosales, C., Weintraub, N., 1987. Canine lymphoma-associated antigens defined by murine monoclonal antibodies. Cancer Immunol Immunother 24, 197-201.

Strong, J.E., Lee, P.W., 1996. The v-erbB oncogene confers enhanced cellular susceptibility to reovirus infection. J Virol 70:612-616.

Strong, J.E., Coffey, M.C., Tang, D., Sabinin, P., Lee, P.W., 1998. The molecular basis of viral oncolysis: usurpation of the Ras signaling pathway by reovirus. EMBO J 17:3351-3362.

Strong, J.E., Tang, D., Lee, P.W.K., 1993. Evidence that the epidermal growth factor receptor on host cells confers reovirus infection efficiency. Virology 197:405-411.

Sun, J., Pedersen, M., Bengtsson, S., Rönnstrand, L., 2007. Grb2 mediates negative regulation of stem cell factor receptor/c-Kit signaling by recruitment of Cbl. Exp Cell Res 313:3935-3942.

Suter, S.E., Chein, M.B., von Messling, V., Yip, B., Cattaneo, R., Vernau, W., Madewell, B. R., London, C.A., 2005. In vitro canine distemper virus infection of canine lymphoid cells: a prelude to oncolytic therapy for lymphoma. Clin Cancer Res 11:1579-1587.

Tai, J.H., Williams, J.V., Edwards, K.M., Wright, P.F., Crowe, J.E.J., Dermody, T.S., 2005. Prevalence of reovirus-specific antibodies in young children in Nashville, Tennessee. J Infect Dis 191:1221-1224.

Takahashi, T., Kitani, S., Nagase, M., Mochizuki, M., Nishimura, R, Morita, Y., Sasaki, N., 2001. IgG-mediated histamine release from canine mastocytoma-derived cells. Int Arch Allergy Immunol 125:228-235.

Takeuchi, Y., Fujino, Y., Fukushima, K., Watanabe, M., Nakagawa, T., Ohno, K., Sasaki, N., Sugano, S., Tsujimoto, H., 2012. Biological effect of tyrosine kinase inhibitors on three canine mast cell tumor cell lines with various KIT statuses. J Vet Pharmacol Ther 35:97-104.

Tan, Y., Demeter, M.R., Ruan, H., Comb, M.J., 2000. BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. J Biol Chem 275:25865-25869.

Tani, N., Dohi, Y., Kurumatani, N., Yonemasu, K., 1995. Seasonal distribution of adenoviruses, enteroviruses and reoviruses in urban river water. Microbiol Immunol 39:577-580.

Taylor-Robinson, D., 1965. Respiratory virus antibodies in human sera from different regions of the world. Bull World Health Org 32:833-847.

Taylor, S.J., Resnick, R.J., Shalloway, D., 2001. Nonradioactive determination of Ras-GTP levels using activated ras interaction assay. Meth Enzymol 333:333-342.

Thirukkumaran, C.M., Luider, J.M., Stewart, D.A., Cheng, T., Lupichuk, S.M., Nodwell, M.J., Russell, J.A., Auer, I.A., Morris, D.G., 2003. Reovirus oncolysis as a novel purging strategy for autologous stem cell transplantation. Blood 102:377-387.

Thirukkumaran, C.M., Nodwell, M.J., Hirasawa, K., Shi, Z.Q., Diaz, R., Luider, J., Johnston, R.N., Forsyth, P.A., Magliocco, A.M., Lee, P., Nishikawa, S., Donnelly, B., Coffey, M., Trpkov, K., Fonseca, K., Spurrell, J., Morris, D.G., 2010. Oncolytic viral therapy for prostate cancer: efficacy of reovirus as a biological therapeutic. Cancer Res 70:2435-2444.

Thirukkumaran, C.M., Shi, Z.Q., Luider, J., Kopciuk, K., Gao, H., Bahlis, N., Neri, P., Pho, M., Stewart, D., Mansoor, A., Morris, D.G., 2013. Reovirus modulates autophagy during oncolysis of multiple myeloma. Autophagy 9:413-414.

Thompson, H., Wright, N.G., Cornwell, H.J.C., 1970. Experimental reovirus infection in dogs: a preliminary report. Res Vet Sci 11:302-304.

Twigger, K., Roulstone, V., Kyula, J., Karapanagiotou, E.M., Syrigos, K.N., Morgan, R., White, C., Bhide, S., Nuovo, G., Coffey, M., Thompson, B., Jebar, A., Errington, F., Melcher, A.A., Vile, R.G., Pandha, H.S., Harrington, K.J., 2012. Reovirus exerts potent oncolytic effects in head and neck cancer cell lines that are independent of signalling in the EGFR pathway. BMC Cancer 12:368.

Umeki, S., Suzuki, R., Shimojima, M., Ema, Y., Yanase, T., Iwata, H., Okuda, M., Mizuno, T., 2011. Characterization of monoclonal antibodies against canine P-selectin glycoprotein ligand-1 (PSGL-1). Vet Immunol Immunopathol 142:119-125.

Umeki, S., Ema, Y., Suzuki, R., Kubo, M., Hayashi, T., Okamura, Y., Yamazaki, J., Tsujimoto, H., Tani, K., Hiraoka, H., Okuda, M., Mizuno, T., 2013. Establishment of five canine lymphoma cell lines and tumor formation in a xenotransplantation model. J Vet Med Sci 75:467-474.

US FDA, 2014 http://www.fda.gov/drugs/resourcesforyou/consumers/ucm143534.htm

Vail, D.M., Pinkerton, M.E., Young, K.M., 2013. Hematopoietic tumors. In: Small animal clinical oncology, Fifth Ed., edited by Withrow, S.J., Vail, D.M., Page, R.L., Saunders Elsevier, St. Louis, MO, USA, pp. 608-687.

van Houdt, W.J., Smakman, N., van den Wollenberg, D.J., Emmink, B.L., Veenendaal, L.M., van Diest, P.J., Hoeben, R.C., Borel Rinkes, I.H., Kranenburg, O., 2008. Transient infection of freshly isolated human colorectal tumor cells by reovirus T3D intermediate subviral particles. Cancer Gene Ther 15:284-292.

Veterinary Cooperative Oncology Group (VCOG), 2011. Veterinary Cooperative Oncology Group - Common Terminology Criteria for Adverse Events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. Vet Comp Oncol 2:195-213.

Vidal, L., Pandha, H.S., Yap, T.A.,White, C.L., Twigger, K., Vile, R.G., Melcher, A., Coffey, M., Harrington, K.J., DeBono, J.S., 2008. A Phase I study of intravenous oncolytic reovirus type 3 Dearing in patients with advanced cancer. Clin Cancer Res 14:7127-7137.

Voldborg, B.R., Damstrup, L., Spang-Thomsen, M., Poulsen, H.S., 1997. Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. Ann Oncol 8:1197-1206.

Waters, S.B., Holt, K.H., Ross, S.E., Syu, L.J., Guan, K.L., Saltiel, A.R., Koretzky, G.A., Pessin, J.E., 1995. Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex. J Biol Chem 270:20883-20886.

Watzinger, F., Mayr, B., Gamerith, R., Vetter, C., Lion, T., 2001. Comparative analysis of ras proto-oncogene mutations in selected mammalian tumors. Mol Carcinog 30:190-198.

Webster, J.D., Yuzbasiyan-Gurkan, V., Kaneene, J.B., Miller, R., Resau, J.H., Kiupel, M., 2006. The role of c-KIT in tumorigenesis: evaluation in canine cutaneous mast cell tumors. Neoplasia 8:104-111.

Weiner, H.L., Fields, B.N., 1977. Neutralization of reovirus: the gene responsible for the neutralization antigen. J Exp Med 146:1305-1310.

White, C.L., Twigger, K.R., Vidal, L., De Bono, J.S., Coffey, M., Heinemann, L., Morgan, R., Merrick, A., Errington, F., Vile, R.G., Melcher, A.A., Pandha, H.S., Harrington, K.J., 2008. Characterization of the adaptive and innate immune response to intravenous oncolytic reovirus (Dearing type 3) during a phase I clinical trial. Gene Ther 15:911-920.

Wilcox, M.E., Yang, W., Senger, D., Rewcastle, N.B., Morris, D.G., Brasher, P.M., Shi, Z.Q., Johnston, R.N., Nishikawa, S., Lee, P.W., Forsyth, P.A., 2001. Reovirus as an oncolytic agent against experimental human malignant gliomas. J Natl Cancer Inst 93:903-912.

Witte, O.N., 1990. Steel locus defines new multipotent growth factor. Cell 63:5-6.

Yamazaki, J., Baba, K., Goto-Koshino, Y., Setoguchi-Mukai, A., Fujino, Y., Ohno, K., Tsujimoto, H., 2008. Quantitative assessment of minimal residual disease (MRD) in canine lymphoma by using real-time polymerase chain reaction. Vet Immunol Immunopathol 126:321-331.

Yancey, D., Nelson, K.C., Baiz, D., Hassan, S., Flores, A., Pullikuth, A., Karpova, Y., Axanova, L., Moore, V., Sui, G., Kulik, G., 2013. BAD dephosphorylation and decreased expression of MCL-1 induce rapid apoptosis in prostate cancer cells. PLoS One 8:e74561.

Yang, W.Q., Lun, X., Palmer, C.A., Wilcox, M.E., Muzik, H., Shi, Z.Q., Dyck, R., Coffey, M., Thompson, B., Hamilton, M., Nishikawa, S.G., Brasher, P.M., Fonseca, K., George, D., Rewcastle, N.B., Johnston, R.N., Stewart, D., Lee, P.W., Senger, D.L., Forsyth, P.A., 2004. Efficacy and safety evaluation of human reovirus type 3 in immunocompetent animals: racine and nonhuman primates. Clin Cancer Res 10:8561-8576.