Oncolytic bluetongue viruses: promise, progress, and perspectives

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Humans are sero-negative toward bluetongue viruses (BTVs) since BTVs do not infect normal human cells. Infection and selective degradation of several human cancer cell lines but not normal ones by five US BTV serotypes have been investigated. We determined the susceptibilities of many normal and human cancer cells to BTV infections and made comparative kinetic analyses of their cytopathic effects, survival rates, ultra-structural changes, cellular apoptosis and necrosis, cell cycle arrest, cytokine profiles, viral genome, mRNAs, and progeny titers. The wild-type US BTVs, without any genetic modifications, could preferentially infect and degrade several types of human cancer cells but not normal cells. Their selective and preferential BTV-degradation of human cancer cells is viral dose-dependent, leading to effective viral replication, and induced apoptosis. Xenograft tumors in mice were substantially reduced by a single intratumoral BTV injection in initial in vivo experiments. Thus, wild-type BTVs, without genetic modifications, have oncolytic potentials. They represent an attractive, next generation of oncolytic viral approach for potential human cancer therapy combined with current anti-cancer agents and irradiation.

Keywords: oncolytic bluetongue viruses, cancer treatment, selective cytotoxic effects, viratherapeutics

Parasites, fungi, bacteria, and viruses are the four major types of human pathogens causing many human diseases with cellular damages and continuous confrontations to our immune systems. Humans tend to dislike their existence. However, recent scientific findings indicated that especially with viruses, the infectious, and pathological outcomes can be converted to some beneficial potential for human health. The potential application of viruses to retard the growth of human cancers was initially introduced by Dock (1904) when he published a landmark article in the American Journal of Medical Science entitled “Influence of complicating diseases upon leukemia.” He had observed and recorded that the white blood cell count of a patient with chronic myelogenous leukemia (CML) decreased dramatically during what was described as a flu-like illness. Thus, he “bravely” made the first suggestion to fellow physicians to use viral infection to treat tumors. The exciting idea of using viruses to destroy cancer cells led numerous medical treatment attempts for over five subsequent decades. However, using only wild-type viruses without genetic modifications such as selective genetic deletion of viral virulent domains or modification of viral genes critical for viral replication and the addition of immune enhancer genes have not been successful. Parallel to this innovative idea was the suggested use of “superbugs” called bacteriophages that kill bacteria but do not harm humans, to combat pathogenic bacteria that kill thousands of people globally each year (Hausler, 2006). The subsequent discovery of phage genetic conversion that might lead to production of either endotoxin and/or exotoxin tempered the use of this medical “superbug” therapy (Singer, 1976) but this approach has begun to resurface in recent years. With the introduction and availability of penicillin to combat bacteria, both medical “superbug” therapy and uses of viral infection to treat and destroy different cancers were largely ignored in the twentieth century (Liu, 2006; Liu et al., 2007). Thus, a century of work on potential uses of oncolytic viruses to treat cancers has not been well accepted and this approach has not yet generated any significant breakthroughs to treat human cancers.

In 1998, Professor Patrick Lee and his associates at the University of Alberta, Calgary provided the first data and evidence that reovirus with a segmented double-stranded (ds) RNA genome could selectively destroy certain human cancer cells in vitro (Strong et al., 1998). This “proof-of-principle” demonstration was a novel breakthrough and has raised hope that reovirus specifically and other viruses (Kim et al., 2006) with oncolytic potentials can be killing machines against human cancers (Kikuchi et al., 1997; Rodgers et al., 1997; Liu, 2006; Holtz, 2007). Through intensive investigation and exploration, the oncolytic potentials of viruses have been discovered in six major viral families, reovirus type 3, papillomaviruses, herpesviruses, hepadnaviruses, flaviviruses, and retroviruses (Norman and Lee, 2005; Alain et al., 2006, 2007; Kim et al., 2006; Shen and Nemunaitis, 2006; Vidal et al., 2006; Burroughs et al., 2007; Qi et al., 2007; Cripe et al., 2009; Ou and Yen, 2010). Since all these, with the exception of reovirus, are also “human cancer viruses” and they cause more than a fourth of all human cancers, innovative methods incorporating recent advances (Wildner, 2003) in molecular biology and genomics/proteomics have been developed and/or modified in the last decade to reduce the pathology of these viruses and to direct and enhance their specific oncolytic activities against different cancer cells and tumors (Aghi and Martuza, 2005; Alain et al., 2006; Holtz, 2007; Ou and Yen, 2010). This generally involves deleting or modifying essential viral genes that restrict the replication of these viruses except in cancer cells. In recent years, combinatorial approaches using RNA interference (RNAi) or the addition of transgenes, whose products target molecules that are over-expressed only or preferentially in cancer cells, have also been formulated with the uses of these oncolytic viruses.
One potential next generation newcomer recently discovered in the oncolytic virus field is the bluetongue virus (BTV), the genomics, and proteomics of which we have worked with for the last 25 years (Kowalik and Li, 1987, 1989, 1991; Li et al., 1987, 1989; Kowalik et al., 1990a,b; Li and Yang, 1990, 1992; Hwang et al., 1992a,b, 1993, 1994; Yang and Li, 1992, 1993; Li and Hwang, 1992; Yang et al., 1992a,b; Hwang et al., 1993, 1995; Hwang and Li, 1993; Hayama and Li, 1994; Wang et al., 1996; Huang and Li, 1997, 2000; Fillmore et al., 2002; Xiao et al., 2004; Hu et al., 2008). BTV belongs to the orbivirus (ring or circle in Greek) genus of the Reoviridae Family which also includes the reovirus. BTVs were initially isolated in several endemic episodes in Africa in late 1960s and early 1970s (Goltz, 1978; Erasmus, 1985; Gorman, 1990). Currently there are 24 different BTV serotypes worldwide. The viral genome of BTVs contains 10 ds-RNA fragments, each of which encodes for one viral structural or non-structural protein (Roy, 1989, 1992) except the S4 fragments that can encode for two non-structural proteins (Wu et al., 1992; Hyatt et al., 1993). BTV transmitted via the blood-feeding midges, Culicoides variipennis in North America and C. imicola in Africa and the Middle East, is the causal agent of bluetongue disease (BD) in domestic cattle and wild ruminants, with virus-induced hemorrhagic fevers (HVFs), vasculitis, edema of the face, lips, muzzle, and ears, excessive salivation, hyperemia of the oral mucosa, and various necrosis and apoptosis of epithelial and mucosal surfaces (Goltz, 1978; Barratt-Boyes and MacLachlan, 1995; Huang and Li, 2000). Neurovirulence has also been detected with some recent BTV isolates (Waldvogel et al., 1987; Carr et al., 1994). With nasal mucopurulent discharges, the tongue becomes cyanotic, and thus, the name, BTV.

Bluetongue viruses (BTVs) are associated with BD in domestic cattle and wild ruminants with much interspecies variability (Goltz, 1978; Gibbs, 1983; Gibbs et al., 1983; Erasmus, 1985; Gorman, 1990), including fatality in sheep and progressive recovery in wild ruminants after BTV infection. Molecular biology efforts from our group (Kowalik and Li, 1987, 1989, 1991; Li et al., 1987, 1989; Kowalik et al., 1990a,b; Li and Yang, 1990, 1992; Hwang et al., 1992a,b, 1993, 1994; Yang and Li, 1992, 1993; Li and Hwang, 1992; Yang et al., 1992a,b; Hwang et al., 1993, 1995; Huang and Li, 1993; Hayama and Li, 1994; Wang et al., 1996; Unger et al., 1988; de Mattos et al., 1991; He et al., 1991; Carr et al., 1994; Odeon et al., 1999), N. J. MacLachlan (Whetter et al., 1990; Rossitto and MacLachlan, 1992; Barratt-Boyes and MacLachlan, 1995; DeMaula et al., 2000, 2001), M. J. Grubman (Campbell and Grubman, 1985; Devaney et al., 1988; Grubman and Samal, 1989; Grubman et al., 1990; Lewis and Grubman, 1990), M. H. Jeggo (Jeggo and Wardley, 1982a,b, 1985; Jeggo et al., 1985, 1986), L. F. Wang (Wang et al., 1988, 1989, 1994, 1996; He et al., 1991; Nagesha et al., 2001), A. M. Wade-Evans (Wade-Evans, 1990a,b, 1992; Wade-Evans et al., 1992, 1996; Pritchard et al., 1995), and others (Shipham, 1979; Moss et al., 1990; Dunn et al., 1991; Cowley, 1992; Harding et al., 1995; Theron and Nel, 1997; Taraporewala et al., 2001), have begun to identify and determine the sequences of each of the 10 BTV genes of many BTV serotypes and the functionalities of the corresponding viral structural and non-structural proteins underlying much of the variability in different animals. The results of viral genomic and protein sequences, their replication cycles in different host cells, multifunction of the structural, and non-structural proteins, etc., can also be found in several BTV reviews (Barber et al., 1985; Campbell and Grubman, 1985; Roy, 1989, 1992, 1996; Eaton et al., 1990, 1996; Gorman, 1990; Roy and Gorman, 1990; Osburn, 1994; Huang and Li, 2000; Roy and Noad, 2006; Yang, 2009).

In our laboratory, we have sequenced the entire viral genomes of BTV 2, 11, 13, and 17, and some of BTV 10 genes (their accession numbers are shown in Table 1), established their genetic relatedness, identified various antigenic epitopes, and functional characteristics of several viral proteins, located some of the nucleic acid binding domains of viral proteins, and recently identified three anti-BTV compounds, and their mechanisms of action (Li et al., 1987, 1989; Li and Yang, 1990, 1992; Hwang et al., 1992a,b, 1993, 1994; Yang et al., 1992a,b; Li and Hwang, 1992; Hwang et al., 1993, 1995; Hwang and Li, 1993; Hayama and Li, 1994; Wang et al., 1996; Huang and Li, 1997; Fillmore et al., 2002; Xiao et al., 2004; Hu et al., 2008).

In addition to our continuous pursuit of the virologic biosystems of BTVs, we have discovered their oncolytic activities that can selectively degrade human cancer cells both in vitro (Xiao et al., 2004; Hu et al., 2008) and in vivo (manuscript in preparation), but not normal human cells. Thus, greater attention is devoted in our lab to further explore and investigate the oncolytic activity of
BTV and identify the viral factors, events, and pathways with which BTVs can selectively degrade human cancer cells. These data can be used for potential treatment of human cancers after additional in vivo and subsequent clinical studies.

**UNIQUE NOVEL ONCOLYTIC ACTIVITIES OF THE BTV ViroLogic SYSTEM**

Bluetongue viruses, pathogenic only to domestic cattle and wild ruminants (Barratt-Boyes and MacLachlan, 1995; Tsuboi and Imada, 1997), are non-pathogenic to humans (Xiao et al., 2004; Hu et al., 2008) and thus, humans do not have pre-existing antibodies to BTVs (Jeggo et al., 1983; Barratt-Boyes and MacLachlan, 1995; Dong et al., 1998). Not only can BTV be grown easily in vitro (Jameson and Grossberg, 1981; Du, 1985; Samal et al., 1985; Sundin and Mecham, 1989; Dong et al., 1998; Wechsler and McHolland, 1998; Chen et al., 1999; Prasad et al., 1999; Lei et al., 2004; Xiao et al., 2004; Liang et al., 2006; Hu et al., 2008), it has powerful oncolytic activity against many different in vitro cultured cancer cell lines (Chen et al., 1999; Xiao et al., 2004, Hu et al., 2008), such as A498 human kidney cells (ATCC: HTB-4), HEP-G2 human liver cells (ATCC: HB-8065), and A549 human lung epithelial cells (ATCC: CCL-185), baby hamster kidney cells (BHK-21), monkey kidney (Vero) cells (Ramig et al., 1989), mouse fibroblast cells (NIH 3T3), SPC-A-1 cells (Lei et al., 2004), MA 782 cells (Liang et al., 2006), and in mouse models. No normal human cells such as the primary human embryo lung fibroblast (HEL) and primary murine embryos fibroblast (MEF) have ever been successfully infected by BTV (Dong et al., 1998; Chen et al., 1999; Lei et al., 2004; Xiao et al., 2004; Liang et al., 2006; Hu et al., 2008) and thus, humans are normally sero-negative. Madin–Darby canine kidney (MDCK) which is sensitive to reovirus apoptosis also proved resistant to BTV infection except in high MOI (unpublished data). BTVs can also infect cultured insect cells (King and Alders, 1985; Samal et al., 1987; Guirakhoo et al., 1995; Mullens et al., 1995; Mertens et al., 1996; Xu et al., 1997; Tan et al., 2001).

In the last decade, our lab and collaborators have used both cell culture systems (Dong et al., 1998; Chen et al., 1999; Lei et al., 2004; Xiao et al., 2004; Liang et al., 2006; Hu et al., 2008) and mice (manuscript in preparation) to investigate and determine the oncolytic activities and capacities of BTVs and their selective degradation of human cancer cells as potential treatment of human cancers. Human cancer cell lines such as Hep-3B and Hep-G2, A549, A498, SPC-A-1, and MA 782 cells infected with BTV 10, 11, 13, 17, and HbC, at various MOIs exhibited the following easily detected characteristics 36 h post-infection (pi) but not in HEL and MEF cells in multiple determinations (Xiao et al., 2004; Hu et al., 2008). Briefly:

1. Between 70 and 90% cytopathic effects (CPE) were readily observed and detected along with cellular apoptosis and necrosis in many human cancer cell lines.
2. Various degrees of morphological and cellular damage could be visualized by either light, confocal, or electron microscopy (EM).
3. Viral particles could be seen intra-cellularly and at the plasma membrane.
4. Cellular dilated endoplasmic reticulum, nuclear chromatin condensation, and cytoplasmic shrinking were readily detected.
5. The survival rate of several human cancer cell lines was low if high MOI was used.
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7. Primary HEL and MEF cells survived very well for 6–9 days independent of the MOI used for testing.
8. Human cancer cells infected with BTVs had more efficient viral replication and provided higher viral progeny yields.
9. Selective cell cycle arrest at sub-G1 peak in several human cancer cells infected with BTV-10 (Hu et al., 2008) and HbC3 similar to the reovirus-induced G2/M cell cycle arrest (Poggioli et al., 2000).

After tumor cells were grown in culture and then injected as xenografts into mice (eight mice/sample), tumors developed and all these mice died in less than 8 days (Preliminary data). When 0.1 ml of BTVs with different MOIs in sterile phosphate-buffered saline (PBS) was injected at three different sites of each xenograft, the sizes of tumors in mice were reduced 60–85%. Most mice with the reduced tumors survived more than 35 days before they were sacrificed for tissue and organ samples. There was also no weight loss (manuscript in preparation). The outcomes of three independent repetitions were similar. Thus, BTV’s selective cytotoxic effects to human tumor cells in vitro and partially in vivo have been clearly indicated and demonstrated. Further in vivo research and clinical studies are now in progress to document and investigate this unique oncolytic potential of BTVs which will provide us better and safer application in humans either in monotherapy or combination therapy in the near future.

**INFLAmMATory MEDIators ARE ELEVATED dURING BTV INFECTION**

Bluetongue viruses administration has been shown to cause cell death and apoptosis in several cell lines as well as in several human tumor cell lines (Wechsler and McHolland, 1998; Xiao et al., 2004;
Liang et al., 2006; Hu et al., 2008). The mechanism by which tumor reduction occurs is still unknown. However, in experiments with cultured human cancer cells, the cells detached and died progressively throughout BTV infection. All BTV genes are transcribed and BTV proteins are expressed during infection.

In the analysis of secreted analyte expression, cytokine and chemokine levels from mock- and BTV-17 infected cells were determined using Multiplex Immunoassay Kits and reagents supplied from Quansys Biosciences (Logan, UT, USA), according to the supplier's protocol. Twenty-five analytes were tested and these included IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFNγ, TNFα, TNFβ, TGF-b1, MCP-1, RANTES, ANG2, HGF, TIMP, TPO, VEGF, PDGF, FGF-basic, and CRP (manuscript in preparation).

Of the 25 analytes tested for during infection, only seven were found to be expressed from five cell lines: A498 human kidney cells, HEP-G2 human liver cells, A549 human lung epithelial cells (ATCC: HTB-4, HB-8065, CCL-185, respectively), BHK-21 cells, or Vero cells. Of these seven analytes, five were found to be consistently elevated upon BTV infection, with the expression pattern consistent among cell lines. These are IL-6, IL-8, MCP-1, RANTES, and FGFβ. The levels of VEGF and TIMP were similar in control and BTV-infected cells. However, A498 kidney cells responded to infection with the greatest secretion of cytokines, followed by A549 lung cells while HEP-G2 liver cells secreted the least amount of cytokines.

Polyinosinopolyctosine (poly I:C) is a synthetic ds-RNA that has been used to assess the role of ds-RNA segments of many ds-RNA viruses in vitro since it can induce differential interferon and cytokine production (Der et al., 1998; Diebold et al., 2003; Chiang et al., 2006). However, poly I:C alone did not cause cell death or have a significant impact on cytokine or chemokine expression in BTV-infected human cancer cell lines. Only IL-6 and IL-8 were found to be significantly elevated during poly I:C treatment. Increases in IL-6 and IL-8 were observed only in kidney cells (A498 and Vero) and were most prevalent in samples treated with 1 μg/ml poly I:C. The other cytokines MCP-1, RANTES, and FGFβ were all at expression levels comparable to the placebo treated cells.

With the identification of some of the antigenic epitopes of various BTV proteins (Wang et al., 1972; Gysen et al., 1985; Fukusho et al., 1987; Gould et al., 1988a, 1994; Grieder and Schultz, 1989; Gysen, 1990; Li and Yang, 1990, 1992; Marshall and Roy, 1990; Mecham and Jochim, 1990; Eaton et al., 1991; Rossitto and MacLachlan, 1992; Du Plessis et al., 1994, 1995; Schoenh et al., 1997; Nagesha et al., 2001), BTV immunity has only been studied partially in the last two decades because of lack of supporting funding and reliable immune assays (Jeggo and Wardley, 1982a,b; Gibbs et al., 1983; Jeggo et al., 1983; Campbell and Grubman, 1985; Stott et al., 1985; Fukusho et al., 1987; Huismans et al., 1987a; Marshall and Roy, 1990; Martyn et al., 1991; Li and Hwang, 1992; Jones et al., 1996, 1997; Lin and Zhou, 1996; Wade-Evans et al., 1996; Odeon et al., 1999; Prasad and Minakshi, 1999; DeMaula et al., 2000; Roy, 2003). With the recent development of more reliable immune assays and microarrays, we have found that BTV can induce inflammatory cytokine expression in immortalized human kidney, lung, and liver cells and subsequently kill the infected cell with cell line-dependent severity. Analysis of results from the cytokine profiles and expression levels in these five cell lines indicated that human tumor cells and tumor reduction might be a result of the combined effects of direct viral induced cell death and recruitment of T-lymphocytes (Jeggo and Wardley, 1982a,b; Jones et al., 1996, 1997) to the tumor through elevated cytokine/chemokine production (Shmulevitz et al., 2005, 2010). Very high levels of pro-inflammatory cytokines were secreted from BTV-infected cells and cytokine levels secreted from each of the seven cell types were directly proportional to the measured death of infected cells. Thus, we hypothesize that the cell death and cytokine expression are related and may be caused by shared signaling pathways or events. These pathways/events are currently under investigation. If these same events occurred in human tumors, BTV would be expected to reduce tumor size in infected patients by directly causing infected human cancer cell death and by inducing cytokine expression from the tumor that would lead to lymphocyte recruitment to the site of the tumor. Thus, BTV has the therapeutic potential in cancer because of its selective ability to cause preferential apoptosis of various human cancer cell lines and tumors.

**PROGRESS AND PERSPECTIVES OF ONCOLYTIC VIRUSES**

Ideal oncolytic viruses should have essential survival data, systemic effect, resist immune system clearance, targeted delivery, and distribution, be highly mobile for intravenous spread, travel to and kill distant metastases, have a high rate of replication to stay ahead of immune system clearance, tolerable toxicity, and side effects, etc. Systemic efficacy is the ability of a virus to find and attack distant metastases. Systemic delivery makes sense in cancer where primary tumors and metastases are dispersed. However, systemic approach in cancer is a big problem because we do not have a good target specific delivery device that would facilitate uptake specifically for certain cells. There is no transport vehicle to target specific cell type yet. Oncolytic viruses can become a radical approach to treat cancer. If this can be achieved, then it would be better than the current and conventional therapies. However, it is very difficult to get enough control patients in clinical trials and to get biopsies from different tissues, organs and tumors after the oncolytic virus treatments to determine the efficacy or survival of most oncolytic viruses. Oncovirologists consider oncolytic virus as a dark horse treatment but not a dead one since they know that many anti-cancer agents appear to be actively effective in lab cultures and animal models but failed in human trials.

Many oncolytic viruses (Norman and Lee, 2005; Alain et al., 2006, 2007; Kim et al., 2006; Shen and Nemunaitis, 2006; Burroughs et al., 2007; Qi et al., 2007; Ribacka et al., 2008; Cripe et al., 2009; Ou and Yen, 2010) that are currently in phase I/II/III human clinical trial status include adenovirus, vaccinia virus, coxsackie virus, reovirus, Newcastle disease virus, herpes simplex virus 1, measles virus, and Seneca Valley virus (Burroughs et al., 2007). In 2007, the only oncolytic virus approved for phase III clinical trials in China was the adenovirus (H101) with E1B deletion produced jointly by the Sunway Biotech of China and Onyx Pharmaceuticals of
Emeryville, CA, USA (Privilege communication). Since mid-2005 to the end of 2010, several other oncolytic viruses have also been approved for phase III clinical trials primarily outside US as briefly described below. Thus, cancer therapy with oncolytic viruses has survived and revived, and this unique approach is poised for a comeback (Aghi and Martuza, 2005; Norman and Lee, 2005; Alain et al., 2006; Liu, 2006; Liu et al., 2007).

The results of the last 10–15 years of investigation generated by researchers have increased interest in oncolytic viruses. Investigators have generated and obtained some genetically modified viruses with greater oncolytic potentials (Aghi and Martuza, 2005; Norman and Lee, 2005; Alain et al., 2006; Liu, 2006; Liu et al., 2007). A significant effort is underway to understand the genetic and mechanistic basis of cancers, selective degradation of cancer cells by these oncolytic viruses, and their effects on downstream cellular events and pathways (Strong et al., 1998; Norman et al., 2004; Vorburger et al., 2004; Aghi and Martuza, 2005; Norman and Lee, 2005; Shmulevitz et al., 2005, 2010; Alain et al., 2006; Liu, 2006; Kim et al., 2007; Liu et al., 2007; Marcato et al., 2007; Hu et al., 2008; Cripe et al., 2009; Hill and Lee, 2010; Ou and Yen, 2010; Thirukkumaran et al., 2010). Increasing attention is being paid to protein families like proteases, caspas, and kinas which play critical roles in cancer.

Current therapeutic approaches for human cancer include the regimen of selective surgery, high dose but cytotoxic chemotherapy with two to three drugs and radiation. These approaches are not very effective to combat the increasing numbers of human cancers. Using oncolytic viruses generates a paradigm shift from current cancer treatment using chemotherapy and radiation which typically destroy both normal and cancer tumor cells. As we learn more about cancer biology and oncolytic viruses, new strategies for treating cancers are rapidly evolving. Uses of oncolytic virus alone or with synergistic anti-cancer drugs or irradiation in human clinical trials are currently under consideration or in progress.

Reovirus leads the field of oncolytic viruses and positive clinical trials are very encouraging (Strong et al., 1998; Norman et al., 2004; Norman and Lee, 2005; Shmulevitz et al., 2005, 2010; Alain et al., 2006; Kim et al., 2007; Marcato et al., 2007; Cripe et al., 2009; Hill and Lee, 2010; Thirukkumaran et al., 2010). Other companies and institutes are not far behind. In mid-May 2010, researchers of the University of Helsinki and Oncos Therapeutics have treated 200 patients with their “modified” GMCSF armed oncolytic adenovirus which exhibited anti-tumor immunity that recruited natural killer cells and induced tumor-specific cytotoxic T-cells with strong efficacy and safety. The media report indicated that its clinical benefit is about 45% according to the RECIST criteria with no grade four to five side effects detected. This represents an extension of their initial investigation in 2008.

With these successful applications and those from other oncolytic viruses, we can now add the simple but oncolytic BTV as a key component to the armamentarium of oncologists to combat and destroy the insidious cancer cells since BTVs have similar characteristics and additional subtle advantages over reovirus and other oncolytic viruses.

### COMPARATIVE VIROTHERAPY AND VIRATHERAPEUTICS OF REOVIRUS (Strong et al., 1998; Norman et al., 2004; Norman and Lee, 2005; Shmulevitz et al., 2005, 2010; Alain et al., 2006; Kim et al., 2007; Marcato et al., 2007; Cripe et al., 2009; Hill and Lee, 2010; Thirukkumaran et al., 2010) AND BTV (Dong et al., 1998; Chen et al., 1999; Lei et al., 2004; Xiao et al., 2004; Liang et al., 2006; Hu et al., 2008)

Bluetongue viruses and reovirus are more advantageous than the genetically modified oncolytic DNA viruses since the integration of viral ds-RNA genome into the host cell genome have not been shown or reported. Since humans are sero-negative to BTVs, potential future use of BTV to degrade human cancer by direct intratumoral injection to degrade the injected tumors will not be inhibited by pre-existing immune components within human cancer cells. Thus, BTV is potentially better than Reovirus in initial oncolytic applications. The current mechanisms of oncolytic Reovirus toward human cancer cells have recently been summarized (Strong et al., 1998; Norman et al., 2004; Norman and Lee, 2005; Shmulevitz et al., 2005, 2010; Alain et al., 2006; Kim et al., 2007; Marcato et al., 2007; Cripe et al., 2009; Hill and Lee, 2010; Ou and Yen, 2010; Thirukkumaran et al., 2010).

Genetically modified reovirus serotype 3, but not types 1 and 2, is a proprietary product called Reolysin produced by Reolytics Biotech, Inc. Data for cancer treatment with this agent from the last decade in animal models and lately in phase I/II human clinical trials have shown encouraging indications of its potential oncolytic activities in cancer cells bearing an activated Ras pathway. Phase III human clinical trials have been approved and initiated in 2009. However, the pre-existing anti-reovirus sera in humans worldwide can trigger greater immune responses against this virus, reducing its efficacy and effectively shutting down its potential oncolytic treatment. Uses of oncolytic BTV have greater advantages over reovirus since no anti-BTV serum is detected in most people globally. Even though BTV injected into tumors can activate dendritic cells for early detection of viral infection, it will take 10–14 days before adaptive immune responses can be generated against BTVs which can eradicate xenografted tumor cells in mice in less than 10 days (preliminary data).

ReoGenics Biotech at Calgary, AB, Canada showed the uses of Reovirus (Reolysin) plus cisplatin, a standard chemotherapy agent in both cultured melanoma cells and a mouse model. The combined uses of Reolysin and cisplatin were much better than either of these two agents alone since they appeared to work synergistically (Abstract of the 4th International Symposium on the “Treatment of Cancers with Oncolytic Viruses” at Scottsdale, Arizona March 15–17, 2007). Furthermore, they had also showed that simultaneous and combined uses of Reolysin and gemcitabine could completely eradicate the transplanted colon cancer in four of the five mice (Abstract of the American Association for Cancer Research (AACR) Annual Meeting in April 2007).

Since mid-April, 2007, Oncolytics Biotech has demonstrated some success in multiple Phase II trials with several medical centers in the UK and US, administering intravenous Reolysin to patients with sarcomas that have metastasized to the lung. Multiple clinical trials for other tumors with the US National Cancer Institute (NCI) were conducted in late 2007. Clinical trials of head and neck
cancers began in mid-August 2010. This will help to determine whether Reolysin can migrate systemically to metastasized tumor sites and exhibit its oncolytic activity.

Oncolytics Biotech has expended more than 65 million dollars and a decade of genetic modifications of Reolysin before human clinical trials were approved. Similar and greater expenditures have also been made by other companies and institutes in the last 12–15 years to develop different oncolytic viruses with tremendous personnel efforts before some clinical trials are approved and conducted. We strongly believe that BTV is the front runner of the next generation oncolytic viruses since it needs no genetic modifications and attenuation, and carries no payloads. It can grow fast and spread easily within many human cancer cells. Since there is no pre-existing neutralizing antibody against BTV in humans, they should be undetected nor immunologically inhibited in the initial injection alone or with anti-cancer agents. In addition to the comparison of the oncolysis of human cancer cells by reovirus and BTV as shown in Table 2, BTV has additional advantages. Briefly:

1. Since BTV does not infect normal human cells, we do not have to suppress the immune system before use. However, it might affect subsequent injections.

2. BTV is harmless to normal human cells which have active p53. However, BTV will attack human tumor cells that have impaired p53.

3. BTV is very stable since it can be stored at pH 8.2–8.4 at 4°C for over 20 years with only 5–10% loss of infectivity (unpublished data). It is also stable at room temperature at this pH for 3–4 weeks. Thus, it can be shipped by air-mail globally without significant loss of its infectivity and oncolytic activities (unpublished data).

4. BTV correlates well with measurement of viral level and tumor responses. Thus, this is a direct effect toward cancer cells. 2-D gel electrophoresis and the 3-D assay (Lam et al., 2007) for the measurement of BTV-induced oncolysis are currently under optimization and further development and adaptation.

5. BTV infection of many cultured human tumor cells has led to the production of many “inflammatory” cytokines such as TNF-alpha and IL-6 which strongly activate the kinase activity of p38 MAPK and ERK1/2. These cytokines also assist the subsequent activation of p38-dependent MAPK and PKR pathways which can bring about the necrosis and apoptosis of cancer cells. This strongly suggests that BTV

| Table 2 | Comparison of oncolytic reovirus and bluetongue virus. |
|---------|----------------------------------------------------------|
| **Reovirus** | **Bluetongue virus** |
| About 100x more efficient replication in RAS-transformed cancer cells | 5,000x more efficient replication in RAS-transformed cancer cells |
| Produce potent and specific CPEs | Similar results |
| About 3x more efficient proteolytic disassembly (uncoating) because more active endosomal and lysosomal proteases (cathepsins B and L) are present in cancer cells | Determination in progress |
| Produce 4x more infectious progeny than non-infectious defective interfering particles | Has caspase-3 induced apoptosis |
| About 9x more caspase-induced apoptosis | Presence of Caspase-independent apoptosis |
| Caspase-independent apoptosis (NA) | Similar results |
| Poly (I:C) did not prevent reovirus infection | Higher levels of pro-inflammatory cytokine production |
| Pro-inflammatory cytokines (NA) | Data Not Available (NA) |
| Reovirus persistently infected Raji cell, did not reduce xenograft tumor in mice and “cure” cells (Raji) | No BTV-resistant human cancer cells have not been detected yet |
| HTR1 cell is a highly reovirus-resistant Fibrosacoma cell derived from HT1080. It has reduced cathepsin B activity and it constrains reovirus oncolysis | Persist in human tumor xenografts in mice through day 30 from a single injection |
| Persist in tumor through days (NA) | Similar preliminary results |
| Toxicity tests are well tolerated | Work in Progress |
| Systemic delivery (some success) | No modification is required |
| Required specific genetic modifications | Humans have no pre-existing anti-BTV sera |
| Humans have pre-existing anti-reovirus sera | No integration of viral genome into host cell genome |
| No integration of viral genome into host cell genome | Work under planning and in progress |
| Have done Phase I–II clinical trials and Phase III is ongoing |

*Reovirus references: Strong et al. (1998), Norman et al. (2004), Norman and Lee (2005), Shmulevitz et al. (2005, 2010), Alain et al. (2006), Kim et al. (2007), Marcato et al. (2007), Cripe et al. (2009), Hill and Lee (2010), Thirukumar and et al. (2010).

*BTV References: Dong et al. (1998), Chen et al. (1999), Lei et al. (2004), Xiao et al. (2004), Liang et al. (2006), Hu et al. (2008).
can prime and direct the immune system of cancer patients against certain solid tumors such as breast and prostate cancer.

6. Repeat screening of over 500 human cytokines using biotin label-based cytokine antibody array is currently in progress and data are under comparative analysis.

7. Construction and rescue of recombinant infectious BTV (Boye and Roy, 2007) expressing the enhanced green fluorescent protein (eGFP), using a protocol similar to one recently published by our lab for human parainfluenza virus type 3 (Roth et al., 2010) will be initiated shortly.

DIFFERENT MECHANISMS BY WHICH ONCOLYTIC REOVIRUS AND BTV CAN SELECTIVELY DEGRADE CANCER CELLS

An activated Ras/RalGEF/p38 pathway is potentially involved with the permissiveness of host cells to Reovirus infection (Norman et al., 2004; Shmulevitz et al., 2005; Alain et al., 2007; Kim et al., 2007; Marcato et al., 2007). It has been shown that the c-K-ras gene inside the lung carcinoma cell line A549 we used has mutation at codon 12 (Valenzuela and Groffen, 1986). Since the Ras/RalGEF/p38 signal pathway inside the A549 cell has been activated, this might allow BTV to degrade human tumor cells efficiently similar to reovirus (Stott et al., 1985; Shmulevitz et al., 2005, 2010; Kim et al., 2007; Marcato et al., 2007).

We have further hypothesized that the genetic alterations/mutations of different human cancer cells, such as signals within a Ras-activated pathway, play a key role in the susceptibility of different oncoviruses in addition to specific viral receptors which might be present in human cancer cells but not in normal human cells. Identification of BTV receptor(s) on the surface of human cancer cells is also currently in progress. Comparison of oncolytic potentials between reovirus and BTV is summarized in Table 2.

With regard to the potential cytotoxic effect mechanisms of BTVs, the GP5 protein of BTV identified by our lab (Li and Yang, 1992; Yang and Li, 1992, 1993; Yang et al., 1992a) has recently been shown to induce or trigger apoptosis in mammalian cell lines (Mortola et al., 2004). Furthermore, the non-structural BTV protein, NS-3, has been shown to be a “viroporin,” the polymerization of which forms pores on the surface of BTV-infected cells, leading to severe cellular leakage, and potential cell death (Han and Harty, 2004). We hypothesize that these two BTV proteins and others are involved with the cytotoxic effect of human cancer cells after BTV infection. Individual BTV gene “silencing” with RNAi to determine the contribution of each of the BTV gene products to oncolysis of human cancer cells are also in progress.

TOLL- LIKE RECEPTOR SIGNALING AND BTVs

The protein Toll (means “weird” in German) was discovered in the fruit fly Drosophila melanogaster in 1980s. It is a transmembrane signal reporter protein that functions primarily in development. However, if mutations in Toll occurred in the fruit fly, the infection by Aspergillus fumigates (a fungus) is lethal. Toll-related molecules involved with human innate immunity and coined as toll-like receptors (TLRs) were then discovered in mid-1990s by Ruslan Medzhitov and Charles Janeway (Medzhitov and Janeway, 2002; Norman et al., 2004). TLRs are molecular pattern recognition receptors and can recognize molecules that are broadly shared by many pathogens. Thus, TLRs pathways play a key role in both innate immunity and normal immune physiology that can sense different pathogens and cancer cells. We hypothesize that some of the TLRs have mutated in many cancer cells.

To determine whether mutated TLRs are involved with the recognition of BTVs, we have initiated the “silencing” of each 1 of the 10 known human TLRs TLR-1–10 using RNAi. Human cancer cell lines, A498 and HEK 239, are transfected with DNA plasmids containing siRNA sequences against human TLRs (Invigogen, CA, USA). Preliminary data indicated that endosome-located TLR-3 which specifically recognizes ds-RNA is potentially involved since cells transfected with this TLR-3 RNAi plasmid produced substantially less BTV 17 and 10 progeny. Cells transfected with TLR-2, 4, 7, 8, and 9 RNAi plasmids produced slightly less viral progeny in these two human cancer cell lines. Similar results are also found in A549 and Hep-G2 cells. Experiments are now underway to determine what types of mutation are in the TLR-3 in these four human cancer cell lines and this might provide a potential explanation of why normal human cells are not infected by BTV because the regular TLR-3 has not been modified nor mutated. Several signal pathways related to TLRs are currently under screening using Pathway Screen Arrays (Qiagen/SABiosciences).

CONCLUSION

Human cancers can be divided into two major types, 90 and 10% of which are solid (carcinoma and sarcoma) or liquid (leukemia) tumors, respectively. There have been widely unrealistic claims of personalized cancer medicine in the last decade. Recent data and epidemiological analysis of many tumors reveal that most FDA-approved cancer therapeutic drugs can only provide small incremental improvement and survival to cancer patients. Companies that make many unrealistic claims for human cancer cures in scientific tabloid media do so strictly for financial reasons since these expensive cancer drugs can generate great revenues via physicians who make drug recommendation and to whom drug companies provide substantial and financial “kick-back” incentives. Interestingly, primary tumors are heterogeneous and they do not kill most cancer patients. However, the 90% of deaths of cancer patients are primarily caused by those cancer stem cells that have metastasized to other regions to form metastatic tumors.

Humans are sero-negative toward BTVs since BTVs do not infect normal human cells. Infection and selective degradation of several human cancer cell lines, but not normal cells, in vitro by five US BTV serotypes have been investigated by our lab for the last few years when BTV oncolytic potential was discovered. We have also recently found that direct single injection of oncolytic BTV into the human tumor xenografts in mice, have led to cytotoxic destruction and preferential lysis of cancer cells within the xenograft but not in normal healthy human or mouse cells (preliminary data). Thus, oncolytic BTVs are safer and more effective than most current cancer treatments. However, we believe that oncolytic BTV is not a “stand-alone” therapeutic and that simultaneous combination of BTV with either radiation or chemotherapy can obliterate the tumors more rapidly and extensively. Cancer patients should have fewer side effects since small dosage radiation and anti-cancer drugs are used for this combined treatment.
Related to the oncolytic BTVs, the following questions have been frequently asked: Is widely curing cancer by oncolytic viruses possible or just hype? What are some of the mechanisms of the selective degradation of human tumors? Will this become a therapeutic modality bridging the lab to bedside? Will the data and results be useful and informative to clinical medicine? Will this scientific innovation transform into health gain for humanity? How “big” is the gap between in vitro/in vivo studies and human application? Is oncolytic BTV a vital alternative for cancer therapy? What are the pathways BTV used to carry out this task? How long would it take to translate BTV oncolysis to medicine? Overall, we believe that the oncolytic ability of BTV represents a significant potential to selectively degrade human cancer cells alone and would even be greater when combined with simultaneous anti-cancer drug and radiation treatment.

Our group and collaborators are currently engaged in several types of investigations with these goals: (1) Study the mechanistic basis of lung, kidney, and liver cancer destruction by BTVs, (2) Examine and determine biological profiles of three types of solid tumors after BTV infection, (3) Correlate the responses of these human cancer cells to changes at the cellular DNA, RNA and protein level, and (4) Construction and rescue of an infectious recombinant BTV expressing the enhanced eGFP using the method that we have developed for the human parainfluenza virus type 3 (Roth et al., 2010) with BTV ds-RNA fragments (Kowalik et al., 1990b; Boyce and Roy, 2007), the success of which can further reveal the oncolytic pathways used by BTVs.

Taking the next step in the investigation of BTV oncolysis is very challenging since no one can individually excel in all the technical, scientific, and clinical avenues involved in this endeavor. Our ongoing in vitro work with different human cancer cell lines will assist us in elucidating the potential mechanisms of the BTV oncolytic activities. No CPE was detected nor viral progeny obtained when several primary human cell lines were infected by BTV. This represents a potential benefit in humans. When xenografts implanted in mice were injected directly with BTV, no pathological damages were detected in any tissue or organs 7–21 days post-infection (preliminary data). Thus, the potential risks of BTV oncotherapy is substantially lower than risks associated with other oncolytic viruses. However, this author sincerely hopes that expert advice and suggestions will be forthcoming from different “champions” after they have read this brief review.

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