Review Article

Stem Cell INVESTIGATION

Targeting cancer stem cells with oncolytic virus

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Abstract: Cancer stem cells (CSCs) represent a distinct subpopulation of cancer cells which are shown to be relatively resistant to conventional anticancer therapies and have been correlated to disease recurrence. Oncolytic viruses utilize methods of cell killing that differ from traditional therapies and thus are able to elude the typical mechanisms that CSCs use to resist current chemotherapies and radiotherapies. Moreover, genetically engineered oncolytic viruses may further augment the oncolytic effects. Here we review the recent data regarding the ability of several oncolytic viruses to eradicate CSCs.

Keywords: Cancer stem cell (CSC); virotherapy; oncolytic

Received: 23 September 2014; Accepted: 14 November 2014; Published: 28 November 2014.
doi: 10.3978/j.issn.2306-9759.2014.11.01
View this article at: http://dx.doi.org/10.3978/j.issn.2306-9759.2014.11.01

Introduction

In recent years, the development of anticancer agents has improved survival of patients with cancer. However, tumor resistance to chemotherapies and recurrence remain a big problem. The cancer stem cells (CSCs), also known as "cancer initiating cells (CICs)" or "cancer progenitor cells", are thought to be responsible for it.

CSCs are immortal tumor-initiating cells that can self-renew and have pluripotent capacity (1). The first evidence of CSCs was provided by Bonnet and Dick in 1997 who found that the $CD34^{+}CD38^{-}$ cells from acute myeloid leukemia (AML) patients could initiate hematopoietic malignancy in NOD/SCID mice (2). In 2003, Al-Hajj *et al*. (3) demonstrated the presence of CSCs in breast cancer, which is the first report of CSCs in solid cancer. Later on, CSCs have been discovered in a variety of cancers, including squamous head and neck cancer, gastrointestinal cancer, colon cancer, melanoma, prostate cancer, brain cancer and so on (4-10). It has been proposed that CSCs may be particularly resistant to chemotherapies and radiation therapies, making them prime candidates as the source of relapse (11). These cells are resistant to standard chemotherapeutics because of their relative quiescent state, thereby rendering cell cycle-dependent drugs ineffective.

CSCs shares similar properties with normal stem cells, which express drug resistance genes, such as the ATP-binding cassette protein efflux pump ABCG2 that plays an important role in protection against conventional chemotherapies. Similarly, many identified CSCs express certain genes, making CSCs resistant to anticancer drugs that are transported by these molecules (12,13). Furthermore, a similar resistance to radiation therapies has been attributed to these CSCs (14). Therefore, targeting CSCs may reduce relapse rates and improve long-term outcome for the patients with many types of cancers (15).

Recently, multiple novel therapeutic ways have been designed with the aim of eliminating CSCs. Among them, oncolytic viruses utilize methods of cell killing, which differ from traditional therapies. Oncolytic viruses are effective against quiescent and drug transporter-overexpressing cells, and thus are able to elude the typical mechanism that CSCs use to resist current chemotherapies and radiotherapies. The use of oncolytic virus as a cancer therapy is based on the observation of tumor regression in the face of natural viral infection (16). Later on, it has been developed for cancer therapy for nearly a century. However, the real potential of it for treating cancers has gained particular attention over the past 15 years with the development of genetic engineering for the viruses (17).

Some of the candidate oncolytic viruses have been entered clinical trials with varying degrees of success. ONYX-015, an oncolytic adenovirus, has been tested in randomized trials and its cousin, H101, has been licensed in China. The original effects of oncolytic virus in cancer cells and CSCs were attributed to a mechanism of action involving direct infection and cell lysis. Either by unlocking tumor antigens or by triggering an immune response to infection, viruses can also act as an immunomodulators or even tumor vaccines. In addition, many later-generation oncolytic viruses have been engineered to strengthen its effect in cancer cells while have less effect on normal cells. Moreover, oncolytic viruses have the ability to target specific features of CSCs such as cell surface proteins, transcription factors, and the CSCs environment (18).

Viruses that have been shown to have the potential of eradicating CSCs include herpes simplex virus-1 (HSV-1), adenovirus (Ads), reovirus, vaccinia virus (VV), myxoma virus (MYXV) and so on. We will highlight recent studies using certain oncolytic viruses against CSCs.

Herpes simplex virus (HSV)

HSV is a double-stranded, enveloped DNA cytolytic virus that has shown promise in targeting a variety of malignancies. The majority of studies with HSV have been conducted in malignant brain tumors, since it is a neurotropic virus (19). However, it is capable of killing different types of cancers including sarcomas, melanomas, colon, breast, lung, prostate, and hepatic tumors (20,21). Deletion or mutation of essential *HSV-1* genes (e.g., $\gamma_1$34.5"neurovirulence gene") required for effective viral replication in normal cells but not cancer cells enables the virus to target malignant cells (22). Efficacy of mutant HSV has been demonstrated preclinical in brain tumors and neuroblastomas which have been reported to contain CSCs (23). Two mutant viruses, G207 and HSV1716 have been used safely without dose limiting toxicities in adult patients with recurrent glioblastoma multiform (GBM) (24,25). G207 contains deletions of both copies of the γ34.5 gene, which is the major determinant of HSV neurovirulence, and has the *Escherichia coli LacZ* gene in its *IPC6* gene (*UL39*), whereby the ribonucleotide reductase needed for replication in non-dividing cells is inactivated.

Recent research has examined the ability of engineered HSV to kill CSCs from different tumors especially from neural tumors. Nestin is an intermediate filament protein expressed in embryonic neuroglial cells and has been used as a CSC marker of brain tumor and neuroblastoma. Taking advantage of differential expression of nestin in glioma cells versus normal astrocytes, Kambara *et al*. developed rQNestin34.5 which expresses ICP-34.5 under control of a synthetic nestin promoter (26). Mahller *et al*. used this rQNestin34.5 virus to infect and kill neuroblastoma CSCs (27). Their study showed that CSCs from a neuroblastoma cell line, LA-N-5, were susceptible to infection by rQNestin34.5 virus. Mice incubated with LA-N-5bulk cells preinfected with rQNestin34.5 showed no flank tumor formation for over 2 months whereas mice injected with saline or control virus-treated cells showed rapid tumorigenesis, suggesting that CSC may be effectively targeted. To further evaluate whether the results found in cell lines can also be found in primary human samples, primary human CSCs were obtained from a patient with neuroblastoma. Two of three CSCs were as susceptible as LA-N-5 cells to oncolytic HSV infection (28). Cheema *et al*. described a murine glioblastoma stem cell (GSC) model that recapitulated tumor heterogeneity, invasiveness, vascularity, and immunosuppressive microenvironment in syngeneic immunocompetent mice. Using this model, they tested a genetically engineered oncolytic HSV that was armed with an immunomodulatory cytokine, interleukin 12 (G47-mIL12). G47-mIL12 not only targets GSCs but also increases IFN-γ release, inhibits angiogenesis, and reduces the number of regulatory T cells in the tumor, suggesting that G47Δ-mIL12 provides a multifaceted approach to targeting GSCs, tumor microenvironment, and the immune system, with resultant therapeutic benefit in a stringent glioblastoma model (29).

Current attempt were also made to combine HSV with chemotherapy or other therapeutic modalities to maximize the efficacy in killing CSCs. Kanai *et al*. used MG18L, a kind of HSV containing a U(S) 3 deletion and an inactivating LacZ insertion in U (L) 39, in combination with phosphoinositide3-kinase/Akt pathway inhibitor for treating human glioblastoma (GBM) stem cells (GSC). The results showed that MG18L synergized with the inhibitor in killing GCSs and glioma cell lines, but not human astrocytes (30). Cheema *et al*. reported a combination therapy with a mutant HSV-G47Δ and low-dose etoposide for killing GBM stem cells. G47Δ is derived from G207. The *IPC47* gene and *US11* promoter are also deleted, whereby growth and antigenicity are enhanced and yet safety is maintained. The combination increased survival of mice-bearing contractile human GSC-derived tumors without adverse side effects (31). The combination of G47Δ with the alkylating agent epidemiologist (TMZ) also acts

synergistically in killing GSCs through virus-mediated manipulation of DNA damage responses (32). These results suggest that oncolytic virus-based combination therapy may be a promising strategy to treat resistant and recurrent GBM.

Besides the efficacy of HSV in CSCs of neural tumors, HSV also showed promising effect on breast CSCs. $CD44^+CD24^{-/low}$ population is considered to comprise breast cancer stem-like cells. HSV G47Δ was found to be highly effective to the $CD44^+CD24^{-/low}$ population *in vitro*. When injected at low multiplicities of infection in mice, G47Δ treatment *in vivo* significantly inhibited tumor growth compared with mock treatment (33). This result demonstrated that HSV is effective against breast CSCs.

Adenovirus (Ads)

Ads is a non-enveloped, non-integrated double-stranded DNA virus that has been studied extensively as an oncolytic therapeutic. Wide-type Ads promotes entry of cells into the G1 phase cell cycle by binding Rb via immediate-early protein E1A and releasing the transcriptional factor E2F (34). Thus it can infect both dividing and non-dividing cells in human. Because many tumor cells harbor defects in the Rb/p16 pathway, conditionally replicative adenoviruses (CRAds) with deletion in *E1A* genes showed tumor selectivity as viral replication was abrogated in normal cells with intact Rb/p16, while its replication was not affected in cancer cells (35).

Most Ads enter into cells through their viral fiber knob binding to the host cell surface coxsackie-Ad receptor (CAR), which is highly expressed on normal epithelial cells but is lacking in many tumor cells. Modification of the viral capsid to change the Ad tropism and enable infection of cancer cells through a CAR-independent mechanism has been a common strategy to overcome the lack of CAR in tumor cells (36). One approach is the creation of chimeric vectors, where the whole fiber or only the knob region is replaced with that of another serotype of Ad. This strategy has led to decreased hepatotoxicity following virus administration attributed to less liver tropism, and increased infectivity of targeted tumor by CAR-independent transduction. Recent studies suggest that chimeric oncolytic viruses may be effective against CICs or CSCs. For example, Eriksson *et al.* showed that capsid-modified E1A mutated Ads, Ad5/3-Δ24, which used the Ad receptor serotype 3 receptor that was highly expressed in tumor cells and Ad5.pk7-Δ24, which entered through heparin sulfate proteoglycans, were able to kill breast CSCs. $CD44^+CD24^{-/low}$ cells, identified as breast CSCs were isolated from pleural effusions of breast cancer patient. These cells could be effectively killed by oncolytic Ads Ad5/3-Delta24 and Ad5.pk7-Delta24. In mice, $CD44^+CD24^{-/low}$ cells formed orthotopic breast tumors while virus infection prevented tumor formation (37).

Another strategy for enhancing viral efficacy is based on the insertion of therapeutic genes into the genome of a modified oncolytic Ads, thereby creating a so-called gene-viral therapy. Gene-viral therapy shares the advantages of gene therapy and virotherapy, which can not only directly kill cancer cells by oncolysis, but also target cancer cells and attack at the cell death signaling, tumor microenvironment and angiogenesis as well. Liu and his group have cloned several individual genes such as *sFlt1*, tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*), second mitochondria-derived activator of caspases (*Smac*), IL-24 etc. into an oncolytic Ads, ZD55, and examined the anti-cancer efficacy of the resulting ZD55-gene(s). The anti-tumor effects of all these ZD55-gene vectors are much better than the respective gene therapy or virotherapy alone. They further explored their strategy to involve the use of two anti-tumor genes; two targeting promoters and two anti-tumor genes in gene-viral therapy. These strategies exerted a much stronger anti-tumor effect than using one gene alone (38). The approach of gene-viral therapy has been used in eradicating CSCs, too. Zhang *et al.* engineered a telomerase-specific oncolytic Ad vector carrying *TRAIL* and *E1A* gene. This Ad showed preferential targeting to radioresistant esophageal CSC-like cells (39). Sasaki *et al.* developed an oncolytic Ads, OBP-301 (Telomelysin), a CRAd with a human telomerase reverse transcriptase (*hTERT*) promoter driving expression of *E1A* and *E1B* linked to an internal ribosome entry site. It was cytotoxic in osteosarcoma cell lines and also suppressed tumor growth in a murine osteosarcoma xenograft model (40). Since telomerase plays an important role in tumorigenesis and it appears to be overexpressed in CSCs compared to other tumor cells, therapeutics targeting telomerase may be a promising agent to eradicate resistant CSCs (41). Our recent study combined the *Beclin-1* gene therapy that induces autophagic cell death with SG511 vector (a new Ad5/11 fiber chimeric CRAd) to generate an oncolytic vector SG511-BECN. It can effectively kill leukemic progenitor cells evidenced by almost complete inhibition of colony-forming in leukemic cells. Furthermore, treatment with SG511-BECN resulted in complete elimination of established tumor xenografts in a mouse leukemia model,

suggesting that CRAds armed with therapeutic transgenes such as *Beclin-1* could eradicate leukemia stem cells (42).

Studies of gene-viral therapy of CRAd in brain tumor CSCs have also been pursued. Using an Ad mutant Delta-24-RGD, a CRAd with the Rb binding region deleted from the E1A gene and an inserted RGD (arginine-glycine-aspartic acid) into the H1 loop of the fiber protein allowing the virus to enter cells via αvβ3 and αvβ5 integrins, Jiang *et al*. demonstrated that xenografts derived from glioma CSC were sensitive to killing by the virus. Furthermore, they showed that Delta-24-RGD-mediated cell death is via an autophagy process (43). Delta-24-RGD is currently in a phase I trial in adults with recurrent malignant gliomas (NCT00805376). To further improve the efficacy of Ads for malignant glioma, Nandi *et al*. developed a novel oncolytic Ads, CRAd-Survivin-pk7, an Ad5 virus with a human surviving promoter to drive E1 expression and polylysine modification in the fiber knob to selectively bind heparin sulfate proteoglycans overexpressed in gliomas. It showed significant toxicity against a panel of passaged and primary CD133 (+) glioma stem cells (44). Another study was conducted by Ahmed *et al*. using CRAd-Survivin-pk7 to test its efficacy in HB1.F3.CD cell line, a US Food and Drug Administration-approved neural stem cell (NSC) line, which is currently employed in human clinical trials. Results showed that antiglioma activity of oncolytic virus-loaded HB1.F3.CD cells was effective against clinically relevant human-derived glioma models as well as a glioma stem cell-enriched xenograft model. HB1.F3.CD NSCs loaded with CRAd-Survivin-pk7 overcome major limitations of oncolytic virus *in vivo* and warrant translation in a phase I human clinical trial for patients with GBM (45). Skog *et al*. first reported using Ad vectors other than the most commonly used serotypes 3 or 5 to target glioma CSCs. They showed that an Ads serotype 16 (Ad16) and chimpanzee serotype 23(Ad CV23) effectively killed both $CD133^+$ and $CD133^-$ cells freshly isolated from primary brain tumors, while Ad5 was the least efficient serotype in primary specimens and was only effective in established cell lines. This study indicated that Ad5 treatment had different effects between primary and culture-adapted cells (46).

Myxoma virus (MYXV)

MYXV is a large, double stranded DNA poxvirus which can cause disease in rabbit. The virus does not infect normal human cells, but is cytotoxic to cancer cells through altered AKT signaling (47). AKT pathway is activated in most cancer cells and has also been implicated in regulating the survival of CSCs following radiation (48). Therefore, MYXV may be an excellent candidate to eradicate CSCs.

Redding *et al*. reported that MYXV effectively infected neuroblastoma CIC cultures isolated by Hansford *et al*. (49). Zemp *et al*. investigated the oncolytic potential of MYXV alone and in combination with rapamycin *in vitro* and *in vivo* using human brain tumor-initiating cells (BTICs). Their study suggested that that MYXV in combination with rapamycin infected and killed both the BTICs and the differentiated compartments of GBM and may be an effective treatment even in temozolomide-resistant patients (50). The inherent ability of MYXV to selectively target cancer cells and spare normal cells makes it a suitable oncolytic virus candidate for *ex vivo* purging of human cancer cells prior to autografts. Rahman *et al*. summarized an *ex vivo* "purging" strategy with MYXV to remove CICs from patient autografts prior to transplantation. MYXV can specifically eliminate cancerous stem and progenitor cells from samples obtained from AML patients, while sparing normal $CD34^+$ hematopoietic stem and progenitor cells capable of rescuing hematopoiesis following high dose conditioning (51).

Reovirus

Reovirus (respiratory enteric orphan virus) is a non-enveloped, double-stranded, segmented RNA virus that has shown potential as an oncolytic, targeted agent. Reovirus is oncolytic in its naturally isolated state and has been in development as a potential cancer therapeutic (Reolysin) (52). Its permissiveness has been shown to correlate with the activation status of the Ras signaling pathways in host cells (53). Since Ras signaling pathway is always upregulated in cancer cells, the capability to deliver the virus systemically makes it an appealing oncolytic virus.

The study examining the effects of reovirus on CSCs was focused on breast cancer (54). CSCs were identified based on $CD24^-$ $CD44^+$ cell surface expression and overexpression of aldehyde dehydrogenase. Marcato *et al*. showed that oncolytic reovirus effectively targeted and killed CSCs of breast cancers *in vitro* and *in vivo*.

Vaccinia virus (VV)

VV is a double-stranded, enveloped DNA virus in the poxvirus family that was first utilized as a vaccination against smallpox and more recently has been shown a promising effect as a cancer therapeutic. Mutated viruses

have a deletion in both copies of the thymidine kinase (TK) genes (55). The TK-deleted virus requires thymidine triphosphate for DNA synthesis which is provided by dividing cells. Therefore, it preferentially replicates in dividing cells and tumor cells.

A few studies highlight the potential of VV to target and kill CSCs. Lun *et al*. tested the recombinant, granulocyte macrophage colony-stimulating factor (GM-CSF)-expressing VV JX-594 against CSCs in a panel of high-grade glioma cell lines. Most cells grown in serum-free medium as neurospheres, free floating clumps of cells thought to be enriched for CSC, were killed by the viruses (56). Wang *et al*. used oncolytic VV, GLV-1h68, in breast cancer stem-like cells. Their study demonstrated that GLV-1h68 replicated more efficiently in cells with higher ALDH1 activity that possessed stem cell-like features than in cells with lower ALDH1 activity. GLV-1h68 selectively colonized and eventually eradicated xenograft tumors originating from cells with higher ALDH1 activity (57).

Other viruses

Other oncolytic viruses have also been shown to specifically target cancer cells. These viruses include: vesicular stomatitis virus (VSV), Seneca valley virus (SVV) and Newcastle disease virus (NDV). Some studies have also been done to investigate their potential of killing CSCs (58).

Future directions

Great progress has been made in the field of oncolytic virotherapy. Several viruses have been translated from laboratory to the clinics. Though the use of various oncolytic viruses for eradicating CSCs has been proven to be a promising way, there are still long distance to be successful applied in the clinic. Most of the preclinical work is done in immune-deficient xenograft model, which cannot approximate either the negative or potentially positive effect of an intact immune system in human. Also strategies to enhance viral delivery, ability of targeting CSCs and reduce the virus clearance are needed to be further explored. Combination therapy with chemotherapeutics, monoclonal antibodies, and small molecular inhibitors will be likely being necessary to eliminate CSCs.

Acknowledgements

Funding: This work was supported by Doctoral Fund of Ministry of Education of China (No. 20120101110010), National Natural Science Foundation of China grants (No. 81070419, and No. 81200384), Zhejiang Provincial Natural Science Foundation of China (No. R2090392), Funds of Science Technology Department of Zhejiang Province (No. 2012C13021-2 and No. 2012C37103), Zhejiang Leading Team of S & T Innovation (No. 2011R50015) and Fund of Health Bureau of Zhejiang Province (No. 2010SSA006).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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doi: 10.3978/j.issn.2306-9759.2014.11.01
Cite this article as: Tong Y, Qian W. Targeting cancer stem cells with oncolytic virus. Stem Cell Investig 2014;1:20.

