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Bioengineering ZIKV-like Exosomes in the Treatment of Drug-Resistant Glioblastoma

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SUMMARY Oncolytic viruses are becoming one of the most promising avenues of therapy against a diverse range of cancers due to inherent tumour specificity. Classically, the difficulty associated with the use of viruses for cancer treatment has been how to minimize off target effects. Currently, several modified viruses, mostly DNA viruses, have overcome this barrier and proceeded to phase III clinical trials with a version of the herpes simplex virus becoming the first oncolytic virus to gain FDA approval. With increased exploration of other viruses in the search for cancer therapeutics it was recently determined that the Zika virus possesses enhanced specificity towards the infection of glioblastoma stem cells (GSCs). As GSCs are responsible for the generation of prevalent, drug resistant, poor prognosis glioblastoma it has been hypothesized that the Zika virus could be utilized to effectively treat glioblastoma however, no further studies have been conducted as of yet. This paper proposes the mechanism behind enhanced Zika infection of GSCs along with a novel virus-like exosome system, the Zikasome, in which the specificity of the Zika virus for GSCs can be integrated with the ability of exosomes to transport therapeutics throughout the body to precisely combat glioblastoma. The goal of this paper is not only to better understand how the Zika virus can be employed to treat lethal glioblastoma but to build upon this proposed virus-like exosome system to develop a customisable virus-exosomal "cassette" that can be used in the treatment of any cancer in which virus specificity exists for. The development of such a system would have a revolutionary impact in the field of cancer treatment as many types of cancer that were previously untreatable could be targeted with a high degree of specificity while bypassing the problem of off target effects associated with the utilization of viruses themselves.

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INTRODUCTION

Oncolytic viruses are genetically engineered or naturally occurring viruses that selectively replicate in and kill cancer cells without harming normal tissue [1]. Recently, this area of research has garnered much success as a modified version of oncolytic herpes simplex virus type 1 was approved as the first FDA approved oncolytic virus cancer therapy with others such as variants of adenoviruses, vaccinia viruses and reoviruses in phase III clinical trials in the USA and Europe [1]. Due to the majority of cancer cells lacking competent antiviral immunity mechanisms, these viruses show great potential in the treatment of many forms of cancer however, many studies fail to produce viruses that do not replicate in wildtype cells [1; 2]. Previously, such difficulties with viral off target effects have been prominent in flaviviruses such as hepatitis C, West Nile fever, yellow fever and dengue viruses and therefore, research into the potential of these viruses to treat cancer have recently rarely been investigated by clinical trials [3].

The most recent example has been the Zika virus (ZIKV), a re-emerging flavivirus that is the causative agent of several neurological disorders. ZIKV has been determined to preferentially infect and kill glioblastoma stem cells (GSCs) relatively to normal neuronal cells [4]. GSCs are a self-renewing, tumourogenic, stem cell-like tumour cell population that maintain proliferation, invasive potential and therapeutic resistant in poor prognosis, prevalent glioblastoma brain cancer. Furthermore, ZIKV infection was determined to significantly increase the life span of tumour-bearing mice through localized viral infection of GSCs [4]. The same results were also seen in a replication attenuated strain of ZIKV providing evidence that ZIKV can be modified and still appears to be an attractive oncolytic therapy against drug resistant glioblastoma. However, the use of ZIKV in glioblastoma treatment has been slowed as the mechanism responsible for ZIKV's tropism against glioblastoma cells remains unknown and a method to utilize ZIKV's specificity while minimizing side effects has not been proposed. Often the approach to achieve cancer cell-specific viral infection and replication has been engineering the viral genome [1; 5]. In this paper, to completely avoid use of ZIKV particles we suggest that the recently discovered specificity of the Zika virus for glioblastoma cancer cells can be instead incorporated onto exosomes to create glioblastoma specific ZIKV-like exosomes (Zikasomes).

Exosomes are 30-100 nm membrane vesicles that are involved in intercellular communication. By transferring functional proteins and nucleic acids between cells, exosomes can facilitate the reprogramming of recipient cells [6]. Recently, exosomes have also been observed to play a role in flaviviral infection as infected cells have been shown to release virus-like exosomes containing viral RNA and proteins capable of infection [6]. Of additional importance to this study, exosomes have been used in the specific transport of drugs and bioactive molecules to cancer cells [7; 8]. Therapeutic exosomes can be created to contain certain membrane receptors and carry specific therapeutic drugs, proteins, nucleic acids or molecules allowing for precise treatment of disease [7]. The incorporation of this technology with the specificity of ZIKV to target and kill GSCs will be proposed in this paper to not only target glioblastoma but also many other types of cancers through the creation of a virus-like exosome customisable cassette system.

RESEARCH QUESTIONS

Substantial efforts have been conducted that involve the use of a wide variety of drugs, such as neutralizing antibodies; in attempt to lessen the overwhelming lethality associated with glioblastoma [9]. Viruses have been determined to successfully treat and provide tumour-specific immunity against many forms of cancer, however questions regarding the safety of such treatment remain largely unanswered [4]. Recently, ZIKV has been determined as a high potential treatment against prevalent, drug resistant, lethal glioblastoma [4]. Despite the initial excitement surrounding this finding, it remains to be determined how ZIKV can be modified and attenuated as a treatment option that can be safely administered to patients. As the issue of safety is inevitably present for all novel oncolytic virus candidates it is of high priority to produce a system that can be safely administered independent of what virus candidate is utilized. The system proposed in this paper combines the ability of exosomes to transport bioactive molecules such as proteins and nucleic acids with ZIKV cellular tropism to produce a highly potent treatment against glioblastoma.

Three questions must be addressed to develop this Zikasome construct that specifically targets and destroys GSCs. First, the mechanism behind how ZIKV is significantly more efficient at infecting GSCs over wild-type glial cells must be deduced. Then it must be determined how this GSC specificity of ZIKV can be manipulated and applied to exosomes. Finally, a proposed mechanism investigating how ZIKV proteins induce apoptosis in GSCs is required to add tumour specific lethality to this system. By addressing these three questions, this article will develop a novel virus based system to combat glioblastoma.

PROJECT NARRATIVE

Overexpression of the Axl receptor and Gas6 TAM ligand as the likely answer to enhanced ZIKV infection of glioblastoma

In order to determine how ZIKV preferentially infects GSCs it is crucial to first examine the mechanisms behind ZIKVs neurotropism. The Brazilian ZIKV outbreak in 2015 led to early hypotheses supporting a correlation between ZIKV infection and increased cases of microcephaly, the development of an abnormally small head due to the damage of developing neural tissue, and also, the autoimmune neurological disorder Guillain-Barré syndrome [11, 12]. Further studies determined that neural stem cells and progenitor cells in culture were susceptible to ZIKV, supporting these hypotheses [12]. By quantifying RNA expression in cells present in the developing brain, such as radial glia and astrocytes, it was elucidated that cells that express high amounts of the receptor protein Axl are highly susceptible to ZIKV infection [13]. Axl is a tyrosine kinase receptor of the Tyro3AxlMer (TAM) family involved in apoptotic cell clearance and innate immunity regulation [14]. Recently, it was discovered that binding of ZIKV to Axl first requires the binding of the TAM ligand Gas6 to phosphatidylserine on the ZIKV envelope and that the inhibition of Axl results in protection against ZIKV infection in otherwise susceptible glial cells [15]. This paper predicts that the Axl receptor plays the same role in the entry of ZIKV into GSCs.

Due to the many current advances in sequencing technologies, it has been discovered that glioblastoma tumours and corresponding GSCs greatly overexpress both Axl and Gas6 [16; 17]. The overexpression of Axl and Gas6 in glioblastoma has been correlated to poor prognosis (<12 years), invasiveness and chemoresistance [18; 16]. Interestingly, it is likely that poor prognosis, therapy resistant glioblastoma, in which a form of therapy is greatly required for, is highly susceptible to ZIKV. As previously mentioned Gas6 and the Axl receptor are crucial for ZIKV infection of neural developmental cells and therefore it is predicted that the overexpression of these two proteins provides ZIKV with an enhanced ability to infect GSCs. As most adult neural cells no longer express high amounts of Axl it is

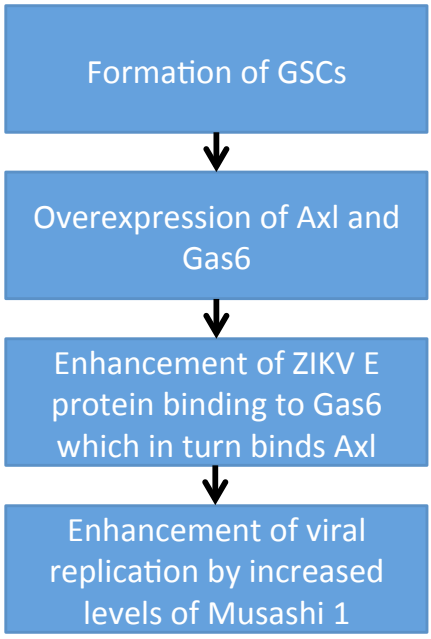

FIG. 1 The proposed mechanism by which ZIKV infection of GSCs is enhanced. The differentiation of neural cells into glioblastoma stem cells is accompanied by the overexpression of Axl, Gas6 and Musashi 1. ZIKV should then bind more easily to these cells, utilizing the ZIKV E protein, as they are expressing high levels of the ZIKV receptor Axl and the adaptor Gas6. Once inside the GSCs higher levels of viral replication are likely seen due overexpression of Mutashi 1.

further predicted that ZIKV would specifically target GSCs in adults suffering from glioblastoma. Additionally, the neurodevelopmental protein Musashi 1 that promotes ZIKV viral replication in developmental neural tissue has also been shown to be overexpressed in GSCs [17]. It is plausible that Musashi 1 overexpression contributes an additive increase in GSC ZIKV infection, when functioning in conjunction with Axl and Gas6 overexpression. Overall, enhancement of ZIKV infection in GSCs is likely due to the overexpression of neurodevelopment markers Axl, and Gas6 (Fig. 1).

The application of ZIKV E protein and Protein S to exosomes for GSC specific targeting

To utilize specificity of ZIKV for GSCs it is important to incorporate the correct component of the virus that allows it to bind to Gas6 and hence Axl into a future anti-glioblastoma system. ZIKV encodes for 10 proteins of which includes 3 structural proteins (the capsid protein (C), envelope protein (E) and pre-membrane protein (prM)) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [19]. Of these 10 proteins, it is the ZIKV E protein that is involved in binding to the host cell receptor during viral attachment and entry [20]. Studies involving E protein crystal structure and cryo-electron microscopy have identified the ZIKV E protein to contain three domains (domains 1,2, and 3) and to associate as dimers on the surface of the virus along with the prM protein [19; 20]. Importantly it has been discovered that the predicted region of interaction with the host cell's receptors is a motif of the E protein called the 150 loop which includes glycans attached to Asn 154 and an unique positive charged patch that surrounds the motif [19; 20]. Therefore, to provide exosomes with the specificity of ZIKV it is important to incorporate this 150 loop motif of the ZIKV E protein onto the exosomal membrane.

Several methods currently exist to accomplish this task such as transfection followed by protein overexpression leading to the enrichment of this protein in the exosomal membrane or alternatively, the bioengineering of the gene of the protein of interest to include an exosomal targeting sequence followed by transfection into the exosome producing cells [21; 7]. The latter approach is suggested in this paper for the creation of the ZIKV-like exosome by fusing the portions of the ZIKV E protein that contain the 150 loop motif of domain 3 along with domain 2, as it is responsible for fusion with the endosomal membrane following clathrin mediated endocytosis of the ZIKV-like exosome, and the flexible region of domain 3, required for the pH dependent conformational change that causes endosomal fusion, with the extra-exosomal N terminus of the lysosomal-associated membrane protein 2b (Lamp2b) (Fig. 2) [6]. Lamp2b is a well-characterized exosomal membrane protein that has been used with a great degree of success in significantly increasing the specificity and cellular uptake of exosomes when fused with membrane proteins for precise exosomal targeting [7; 22; 23]. Importantly, it must be determined if the other domains of the ZIKV E protein such as domain 1, the rest of domains 2 and 3 can be omitted from the plasmid construct without disrupting E protein structure as these domains have been associated with antibody neutralization which would drastically compromise the ability of Zikasomes to circulate to the brain and binding to GSCs [24]. To further reduce immunogenicity, the proposed choice of exosome producing cells are patient derived immature dendritic cells as exosomes produced by this cellular system lack classical surface immunostimulatory markers CD40, CD86, MHC-1 and 2 [25]. These exosomes have also passed multiple phase 1 trials in the use of cancer treatment further promoting their use in the creation of Zikasomes [26].

FIG. 2 Predicted structure of the chimeric ZIKV E protein with the Lamp2b N-terminus exosome localization sequence. The four different regions of the ZIKV E-Lamp2b N terminus protein are shown here. The E 150 loop is involved in receptor binding to the host cell Gas6 and Axl receptor. E Domain II is required for fusion of the exosome with the host cells endosomal membrane. E Domain III is required to the acid dependent conformational change required for endosomal fusion. The Lamp2b N terminus sequence is required for incorporation of this protein into the exosome membrane during Zikasome biosynthesis.

Next, when creating the Zikasome it is important to conclude if the ZIKV prM protein is required for proper orientation of the ZIKV recombinant E protein in the exosomal membrane. Currently, the topic is under debate with several studies stating the need for the prM's chaperone activity in moving the ZIKV E protein throughout the secretory pathway while other studies have documented wild type E organization in the viral envelope without the presence of prM [24]. Lastly, as is common with flaviviruses, the ZIKV E protein can bind multiple cellular receptors involved in different cell types; therefore an extra receptor should be fused to Lamp2b and expressed by the plasmid. The ligand suggested is a membrane-tethered version of another Axl ligand, Protein S [15; 27]. By tethering the soluble Protein S to Lamp2b an additional GSC-specific ligand can be added to the ZIKV-like exosome increasing the ability of the exosome to target GSCs.

The ZIKV NS4A, NS3, NS5, E and C proteins are candidates in the activation of Digoxin induced GSC apoptosis

As mentioned previously the usefulness of exosomes in the treatment of cancer stems from the ability of exosomes to be loaded and then cargo specific drugs or bioactive molecules to the cancerous tissue itself. The application of exosomal cargoing must be utilized in the Zikasome system in order to destroy GSCs, however it is well established that these cancerous cells are resistant to almost all current drug therapies, even the standard-of-care in glioblastoma treatment, temozolomide [16]. An avenue that offers some hope is the mechanism behind how ZIKV can effectively induce apoptosis in GSCs [4]. Previously, the induction of apoptosis of cancer cells through modified exosomes has been utilized in the treatment of lung and ovarian cancer cells [28]. Therefore, once determined the mechanism by which ZIKV induces apoptosis in GSCs can be incorporated into the Zikasome system. Currently, the important link between ZIKV infection of GSCs and apoptosis is predicted to be the recently discovered mammalian Digoxin molecule [28].

Digoxin is a cardiac glycoside, a group of sterol lipids that are well known as secondary metabolites in plants and only recently discovered to be endogenous in mammals, that is a potent inhibitor of the Na^+-K^+-ATPase ion pump [29]. Since the discovery of endogenous cardiac glycosides has occurred it has been determined by both in vivo and in vitro studies that the presence of these lipids increase cancer cell death in a variety of cancers making them potential therapeutics in cancer treatment [30]. Of relevance of this study, Digoxin has been shown previously to be both synthesized in ZIKV infected GSCs and induce apoptosis in wild-type GSCs by caspase-3, which is consistent with the observed mechanism of ZIKV induced apoptosis [4; 28]. Interestingly, Digoxin was further determined to be expressed in ZIKV infected GSCs but not wild type human non-progenitor cells suggesting a glioblastoma specific pathway involving Digoxin and ZIKV [28]. Therefore, it could be predicted that if the ZIKV-like exosomes specified in this paper carried the ZIKV protein or proteins responsible for Digoxin synthesis in glioblastoma these exosomes would not only have specificity in glioblastoma targeting but also in the destruction of GSCs. Such specificity would be predicted to drastically lower off target effects creating a safe, effective treatment option for a form of cancer that currently is associated with poor prognosis.

The cellular synthesis of Digoxin is predicted to be critical in ZIKV induced GSC apoptosis yet it is unknown what ZIKV protein triggers this synthesis. Of the ten ZIKV proteins produced during infection it is the C and NS5 proteins that have been found to be imported into the host cell's nucleus [31]. It is possible that by entry into the nucleus either of these ZIKV proteins could enhance the expression of Digoxin biosynthesis proteins such as Hydroxymethylglutaryl (HMG)-CoA reductase and other proteins of the mevalonate pathway that produces sterols; however little is known about how the NS5 or C proteins act within the nucleus [31]. Additionally, it has been characterized in the dengue virus (DENV), a closely related virus to ZIKV, that the phosphorylation levels of HMG-CoA reductase are reduced during viral infection leading to increased HMG-Coa reductase activity and cellular cholesterol levels [32]. Recently, the DENV proteins NS3, NS4A and E have all been seen to co-localize with HMG-CoA reductase and it's associated kinase, AMP-activated protein kinase, predicting a functional interaction between these proteins [33]. In conclusion, more research must be conducted to determine the connection between ZIKV and Digoxin synthesis in GSCs before ZIKV proteins are transfected into immature dendritic cells to be

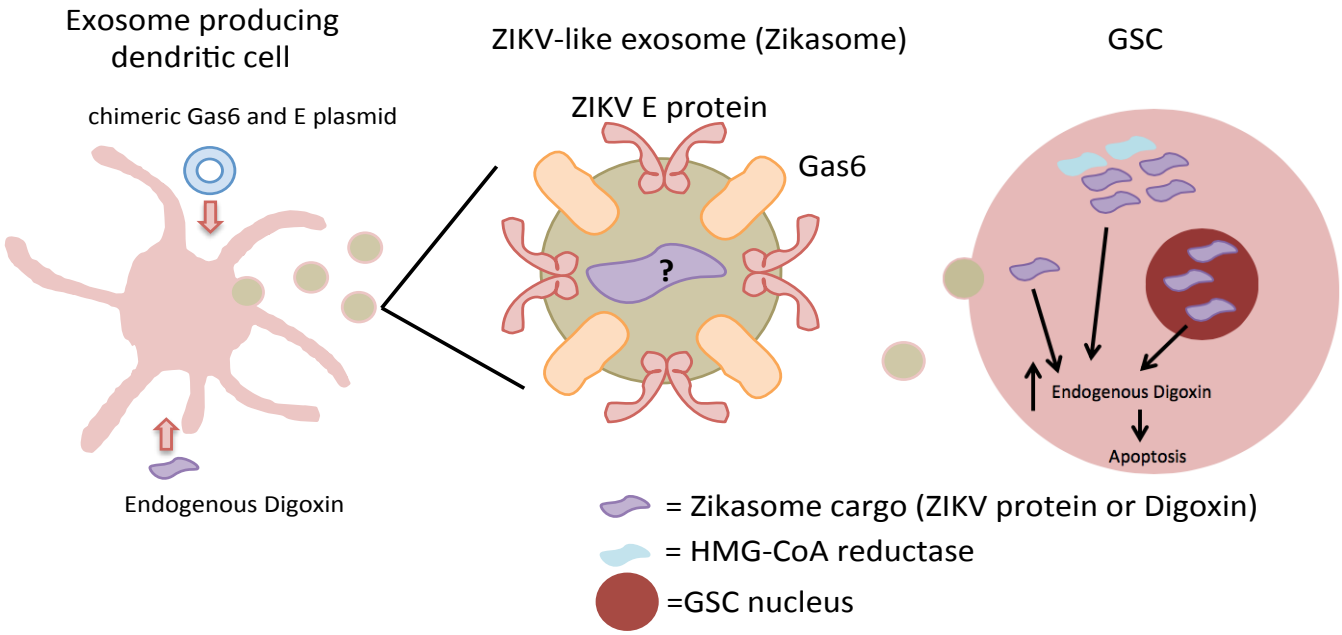

FIG. 3 The production of ZIKV-like exosomes and the mechanism of targeted GSC death. The production of ZIKV-like exosomes occurs when an immature dendritic stem cell is transfected with a plasmid containing both chimeric ZIKV E and Gas6, which are bound to the membrane by the N terminus of the exosomal targeting sequence of Lamp2b. The addition of cargo to the ZIKV-like exosome either involves cells being treated with molecules, such as Digoxin, or ZIKV proteins being overexpressed from the transfected plasmid. If ZIKV proteins are loaded they could either travel to the nucleus and up regulate the expression of genes involved in making Digoxin whereas it is predicted that several ZIKV proteins could enhance HMG-Coa reductase activity leading to Digoxin synthesis. An increase in endogenous Digoxin results either by host cell synthesis or release from the Zika-like exosome, which leads to apoptosis of the GSC.

transported to GSCs, however, it is suggested that currently Digoxin itself should be loaded as cargo into these ZIKV-like exosomes due to its anti-glioblastoma properties (Fig. 3). This could be done by a multitude of techniques such as electroporation, extrusion or sonication however each of these techniques could compromise the chimeric ZIKV E and Protein S protein organization on the exosomal membrane [26]. Therefore, the method of choice in this paper is incubating chimeric E and Protein S transfected immature dendritic donor cells with Digoxin as this technique will incorporate Digoxin into exosomes while maintaining the exosomal membrane structure [26].

SUMMARY AND CONCLUSION

Oncolytic virus therapy has become one of the most promising therapeutic avenues in the treatment of cancer. With rapid advances in molecular biology and genetics many recombinant viruses have been developed and are now undergoing clinical trials however, the largest hurdle in this area of research remains how to engineer viruses that are specific to only cancerous tissue and not wild type host cells. This paper proposes a novel virus-like exosome system that allows for the specific targeting and destruction of cancer cells. To showcase this system the example of ZIKV and its specificity for glioblastoma stem cells, the regenerative cells of glioblastoma, is displayed.

Every year 5 to 8 per 100000 individuals develop drug resistant, lethal glioblastoma in which the associated life expectancy is 9 to 15 months [17]. It was recently discovered that ZIKV specificity infects and destroys the drug resistant glioblastoma stem cells that maintain glioblastoma [4]. To apply this novel finding to the field of glioblastoma treatment it was proposed that the specificity of ZIKV for GSCs be incorporated into an exosome based system. This paper considered three questions that required answers before the ZIKV-like exosome (Zikasome) could be fully envisioned.

First, it was predicted that ZIKV specificity for GSCs results from increased expression and activation of tyrosine kinase receptor Axl and TAM ligand, Gas6 in glioblastoma. Next,

the groundwork for the Zikasome was formed by determining that incorporation of a chimeric version of the ZIKV E protein, containing the exosomal targeting sequence of the Lamp2b protein along with known motifs and domains of the E protein required for host cell binding, and a membrane tethered version of the Axl ligand protein, Protein S, could potentially give exosomes a high degree of specificity for GSCs. Further, by producing the exosomes from immature dendritic cells a low level of immunogenicity and high level of exosomal quality can be maintained which is necessary for patient use. Lastly, the molecule Digoxin was chosen to be loaded into the Zikasome due to its ability to cause apoptosis in GSCs. However, determining which ZIKV protein is responsible for Digoxin synthesis in GSCs would provide Zikasomes with another level of glioblastoma specific targeting.

Overall, by incorporating tumour specificity often characterized in viruses with the ability of exosomes to carry drugs or bioactive molecules that can destroy cancer cells a cancer type specific treatment can be developed. The long term vision of this paper is to develop a "cassette" system where by use of an established database that provides information on which viruses target which types of cancers, and what molecules or drugs can kill these cancer cells a specific virus-like exosome can be produced by matching the viral receptor and molecule that can correctly target and combat this patient's cancer. Such technology would be extremely important in the field of cancer therapeutics, as it would potentially allow for the safe treatment of many forms of cancer, even forms that are tough to treat such as glioblastoma. A current downside to this system is how time consuming it is to produce a large number of exosomes however; by choosing low immunogenicity exosomes they will maintain a longer lasting effect in the body and therefore will require lower doses for effective treatment. Further, this system could also be applied to novel higher yield techniques that are being developed such as cell-derived nanovesicles [35]. In conclusion, the use of the Zikasome in combatting glioblastoma is promising but must first be determined experimentally. Positive results obtained from Zikasomes would validate the novel idea of fusing viruses with exosomes to produce safe, cancer-specific treatment.

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