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Zebrafish encoded 3-O-sulfotransferase-2 serves as receptor during HSV-1 entry and spread

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Abstract

Previously we reported the role of zebrafish (ZF) encoded glucosaminyl 3-O sulfotransferase-3 (3-OST-3) isoform in assisting herpes simplex virus type-1 (HSV-1) entry and spread by generating an entry receptor to HSV-1 envelope glycoprotein D (gD). However, the ability of ZF encoded 3-OST-2 isoform to participate in HSV-1 entry has not been determined although it is predominantly expressed in ZF brain, a prime target for HSV-1 to infect and establish life long latency. Here we report the expression cloning of ZF encoded 3-OST-2 isoform and demonstrate HSV-1 entry into resistant Chinese hamster ovary (CHO-K1) cells expressing the clone. Additional significance of ZF encoded 3-OST-2 receptor was demonstrated using medically important isolates of HSV-1. In addition, interference to HSV-1 entry was observed upon co-expression of HSV-1 gD and ZF 3-OST-2. Similarly HSV-1 entry was significantly inhibited by the pre-treatment of cells with enzyme HS lyases (heparinase II/III). Finally, ZF-3-OST-2 expressing CHO-K1 was able to fuse with HSV-1 glycoprotein expressing cells suggesting their role in HSV-1 spread. Taken together our result demonstrates a role for ZF 3-OST-2 in HSV-1 pathogenesis.

Abbreviations

Zebrafish; heparan sulfate; herpes simplex virus type-1; 3-O- sulfotransferase-2; virus-cell interaction

1. Introduction

Heparan sulfate (HS) is a complex sulfated polysaccharide representing a glycosaminoglycan (GAG) of repeating disaccharide units of *N*-acetylglucosamine and glucuronic/idouronic acid [1]. They are expressed on cell surface as a hybrid molecule of heparan sulfate proteoglycans (HSPGs) covalently linked to a specific core protein

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mediating diverse range of crucial functions during vertebrate and invertebrate development. These functions include cell adhesion, cell signaling, blood coagulation, wound healing, and growth-factor mediated proliferation [2, 3]. The fine structures of HS chain get modified in a complex series of steps involving enzymatic actions such as: *N*-deacetylation/*N*-sulfation of the glucosamine unit, C5 epimerization of the glucuronic acid to iduronic acid, and *O*-sulfation of both residues. The last and the rare *O*-sulfation step involves 3-*O* sulfotransferases (3-OSTs) enzyme, which exist in multiple isoforms [4, 5]. In each of these modification steps, only part of the substrate is modified resulting in a high sequence diversity or heterogeneity which is thought to give HSPGs their functional specificity and versatility [1, 4]. Thus, each 3-OST can potentially generate unique protein-binding sites within the HS chain [4]. HS moieties prominently exposed on the cell surface have also been implicated as primary receptors for human herpesviruses and many other medically important viruses [5–7].

In recent years, Zebrafish (ZF) has become a favorite model organism for biologists not only to study developmental biology but also to understand host-pathogen interactions including inflammation and innate immune response to infectious diseases [8–10]. Likewise, there is a growing literature on the use of ZF as a model to study viral diseases including herpes simplex virus [11–16]. Interestingly ZF is known to express multiple isoforms of heparan sulfate modifying enzyme 3-*O*-sulfotransferase (3-OST) [17–19]. Cadwallader and Yost [18] reported *in vivo* characterization of eight 3-OST family members in ZF with seven genes showing homology to known 3-OST genes in mouse and humans. The diversity in the expression of 3-OST family members in ZF provided us a rationale to examine the role of 3-OST isoforms in HSV-1 entry. Our previous work demonstrated the role of ZF 3-OST-3 in HSV-1 entry and spread similar to human isoform of 3-OST-3 [12]. In this study we aimed to identify a role for ZF encoded 3-OST-2 isoform in HSV-1 entry and spread. The results presented below demonstrate that resistant Chinese hamster ovary (CHO-K1) cells expressing ZF 3-OST-2 are susceptible to HSV-1 entry and spread. The characterization of ZF encoded 3-OST-2 as a generator of a novel receptor for HSV-1 will further aid to the use of ZF as a model to study HSV-1 infection.

2. Materials and methods

2.1. Plasmids

ZF encoded 3-OST-2 gene was cloned into pCDNA3.1 plasmid (Genscript). Human 3-OST-3 expressing plasmid (pDS43) and 3-OST-2 was generous gifts from Dr. Deepak Shukla (University of Illinois at Chicago) [6]. The HSV-1 (KOS) glycoprotein expressing plasmids used were pPEP98 (gB), pPEP99 (gD), pPEP100 (gH), and pPEP101 (gL) [20]. Other plasmids used in this study include pMW13 (RID1 gD), pCAGT7 (T7 RNA polymerase), pT7EMCLuc (luciferase gene) for the luciferase assay, and a control empty vector pCDNA3.1 from Invitrogen (Carlsbad, CA, USA).

2.1. Cell culture and viruses

Wild-type Chinese hamster ovarian-K1 (CHOK1) cells were kindly provided by P.G. Spear (Northwestern University, Chicago). All CHO-K1 cells were grown in Ham's F-12 medium

(Gibco/BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), and penicillin and streptomycin (Gibco/BRL). The β -galactosidase expressing recombinant HSV-1 (KOS) gL86 was provided by P.G. Spear (Northwestern University, Chicago).

2.3. HSV-1 viral entry assay

Previously defined entry assay was used [6]. CHO-K1 cells were grown in 6-well plates to subconfluence and transfected with 2.5 μ g of human or ZF encoded 3-OST isoforms (3-OST-2), HSV-1 gD or pCDNA3.1 using LipofectAMINE (Gibco/BRL). At 16 h posttransfection, the cells were replated into 96-well dishes for infection with recombinant virus. After 6-h post infection, β -galactosidase assay were performed using either a soluble substrate o-nitrophenyl- β -D-galactopyranoside (ONPG; ImmunoPure, Pierce) or X-gal (Sigma). For the soluble substrate, the enzymatic activity was measured at 410 nm using a micro-plate reader. For X-gal assay, the cells were fixed (2% formaldehyde and 0.2% glutaraldehyde) and permeabilized (2 mM MgCl₂, 0.01% deoxycholate, and 0.02% nonidet NP-40 (Sigma). Finally, 1 mL of β -galactosidase reagent (1.0 mg/ml X-gal in ferricyanide buffer) was added to each well and incubated at 37°C for 90 min before the cells were examined using bright field microscopy under the 20 $\times$ objective (Zeiss, Axiovert 100M). Heparinase-II and -III were obtained from Sigma–Aldrich Chemical.

2.5. HSV-1 gD-mediated interference assay

Entry assay was slightly modified for the gD-mediated interference assay [21]. CHO-K1 cells were co-transfected with ZF and or human encoded 3-OST expression plasmid and either gD-expressing or empty vector pCDNA3.1 in a 1:1 ratio, with each plasmid added at a final concentration of 2.5 μ g/ μ l keeping the total amount of transfected DNA constant. After transfection, the cells were infected, and the infection was quantitated by addition of the substrate of β -galactosidase, ONPG, as described above.

2.6. Cell fusion assays

A cell-to-cell fusion assay described previously was used [22]. CHO-K1 cells were grown in 6-well plates to subconfluent levels. The so-called “target” cells were transfected with plasmids expressing 3-OST isoform (either human or ZF 3-OST-2) and the luciferase gene. The “effector” or virus-like cells were co-transfected with plasmids expressing HSV-1 glycoproteins gB, gD, gH, and gL, and T7 RNA polymerase. In either case, the total amount of DNA used for transfection was kept constant. After 16 h, target and effector cells were mixed in a 1:1 ratio and then replated in 24-well dishes. The activation of the reporter luciferase gene as a measure of cell fusion was examined after 24 h. To demonstrate sensitivity to heparinase treatment target CHO-K1 cells expressing ZF 3-OST-2 were treated with a 1:1 mixture of heparinase-II/III for 2 h prior to mixing with the effector cells. Target cells were mock treated with the buffer alone to serve as a control.

3. Results and discussion

3.1. Expression of Zebrafish (ZF) encoded 3-OST-2 enhances susceptibility to HSV-1 entry

We began our study by cloning the open reading frame of ZF encoded 3-OST-2 into pCDNA3.1 for mammalian expression. The inserted sequence was verified by enzymatic

digestion with restriction endonucleases (Fig. 1A and Fig. 1B). In order to determine the ability of ZF encoded 3-OST-2 to mediate HSV-1 entry, CHO-K1 cells were transiently transfected with ZF-3-OST-2 expression plasmid. Entry assays were performed to determine whether the expression of ZF-3-OST-2 rendered CHO-K1 cells susceptible to HSV-1 entry. Using a reporter HSV-1 virus (HSV-1 KOS gL86) the dose response curve for HSV-1 entry was determined and compared with the cells expressing human 3-OST-2 and 3-OST-3 isoforms as controls. In addition, an empty vector, pCDNA3.1. was used as the negative control. As shown in Fig. 2A there was a significant increase in viral entry in CHO-K1 cells-expressing both human and ZF encoded 3-OST-2s relative to the CHO-K1 cells that were transfected with empty vector alone. The ONPG result was further confirmed by X-gal assay. As shown in Fig. 2B, X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactosidase) activity was found to be positive for ZF-3-OST-2 cells (Fig. 2B; panel b) similar to human-3-OST-2 and 3-OST-3 cells (Fig. 2B; panels a and c). Wild type CHO-K1 cells expressing pCDNA3.1 empty vector remained resistant to HSV-1 entry (Fig. 2B; panels a1–c1).

3.2. Zebrafish 3-OST-2 mediates HSV-1 entry of medically relevant isolates

The next question was to evaluate the broader significance of ZF encoded 3-OST-2 receptor. We therefore, tested the entry of different clinical virulent strains of HSV-1 (Patton, 17, and HFEM [23] along with wild-type KOS (WT) and syncytial strain KOS-804 into cells expressing 3-OST-2 receptor. CHO-Ig8 cells that express β -galactosidase upon viral entry [24] were transfected with human and ZF encoded 3-OST-2 expression plasmids. The above listed virulent clinical HSV-1 strains were used to infect the cells. The results from the experiment again showed that ZF 3-OST-2 allowed entry of different HSV-1 strains in a dosage dependent manner as evident by ONPG assay (Fig. 3A).

3.3. Co-expression of glycoprotein D (gD) with ZF 3-OST-2 renders resistance to HSV-1 entry

The interaction between 3-OS HS generated by ZF 3-OST-2 and HSV-1 glycoprotein D (gD) was evaluated by using a previously described gD-mediated interference assay [21]. This assay is based on the principle that cells co-expressing gD and its receptor can endogenously sequester the receptor thereby resulting in cells resistant to HSV-1 entry. To carry out the assay, cultured CHO-K1 cells were transiently co-transfected with an HSV-1 gD-expressing plasmid (or an equal amount of the empty vector, pCDNA3.1. as a control) along with a ZF 3-OST-2 plasmid, followed by infection with serial dilutions of β -galactosidase-expressing HSV-1(KOS) gL86. As shown in Fig. 3B the co-expression of HSV-1 gD with ZF 3-OST-2 resulted in significant loss of HSV-1 entry.

3.4. Zebrafish 3-OST-2 mediated HSV-1 entry is HS dependent

In order to strengthen the role of cell surface expression of 3-O sulfated heparan sulfate (3-OS HS) responsible for cell fusion not the enzyme itself we evaluated the effect of enzymatic removal of 3-OS HS on HSV-1 entry by treatment with a mixture of heparinase-II and -III (1.5U ml/L). These enzymes selectively degrade HS chains by cleaving them [25]. For this experiment, CHO-K1 cells expressing ZF 3-OST-2 were mock-treated or pretreated with heparinase-II/III before infecting with reporter β -galactosidase expressing

HSV-1 gL86 [6]. As presented in Fig 4A heparinase treated cells showed significant reduction in HSV-1 entry (white bar) compared to mock treated cells (black bar). The above results were further confirmed by the X-gal assay using HSV-1gL86 reporter virus. Again the heparinase treated CHO-K1 cells expressing ZF 3-OST-2 showed significantly reduced number of blue cells (Fig. 4B, upper panel) compared to mock treated ZF-3-OST-2 expressing cells (Fig. 4B, lower panel). The results indicate that heparinase treatment degrades HS including 3-OS HS on the cell surface thereby inhibiting HSV-1 entry.

3.5. ZF 3-OST-2 contributes to cell-to-cell fusion with HSV-1 glycoprotein-expressing cells

Finally, the role of ZF 3-OST-2 generated receptor was examined to determine whether the same receptor could also facilitate cell-to-cell fusion. Wild type CHO-K1 cells that are also resistant to virus-induced cell fusion due to absence of a gD receptor were used [6]. To quantify the HSV-1 induced cell fusion between 3-OS HS cells modified by 3-OST-2 and HSV-1 glycoproteins a luciferase reporter gene assay was performed [20, 22]. Wild type CHO-K1 cells were transiently transfected with each of four glycoprotein plasmids: pPEP98 (gB), pPEP99 (gD) pPEP100 (gH), and pPEP101 (gL), as well as, the plasmid pT7EMCLuc that expresses a luciferase reporter gene was considered “effector” cell. In parallel “target” cells were transfected with a 3-OST plasmid expressing ZF encoded 3-OST-2 and the plasmid pCAGT7, which expresses T7 RNA polymerase to induce expression of the Luciferase gene. For a negative control, cells were transfected with T7 RNA polymerase and control plasmid pCDNA3.1. The cells expressing human 3-OST-2 and T7RNA polymerase served as a positive control. As shown in Fig. 5 a high amount of fusion occurred in both human and ZF encoded 3-OST-2 expressing cells (black bars) compared to the negative control (grey bar). Clearly, the 3-OS HS generated by ZF encoded 3-OST-2 is capable of mediating cell fusion as well. These results reinforce our findings that cells expressing ZF 3-OST-2 allow cell fusion to occur, and thus potentially could facilitate spread of HSV-1 in a ZF model.

3.6. Zebrafish 3-OST-2 mediated HSV-1 glycoprotein induced cell-to-cell fusion was HS dependent

In order to strengthen the role of cell surface expression of 3-OS HS responsible for cell fusion not the enzyme itself we evaluated the effect of enzymatic removal of 3-OS HS on cell fusion by treatment with a mixture of heparinase-II and -III (1.5 U ml/L). These enzymes selectively degrade HS chains by cleaving them. For this experiment, both human and ZF 3-OST-2 expressing CHOK1 cells were separated into two distinct pools, one treated with heparinase-II/III and the other untreated and mixed with the equal amount of effector cells expressing HSV-1 glycoproteins and co-cultivated. As seen in Fig. 5 cells expressing either human or ZF 3-OST-2 treated with heparinase-II/III showed about a 90% reduction in fusion (Fig. 5; white bars).

3.7. Discussion

Recently Zebrafish (ZF) was recognized as model system to study neuronal damages during HSV-1 infection [11]. Interestingly ZF is known to express multiple isoforms of HS modifying enzyme 3-OSTs for their physiological development including 3-OST-2 [18].

Our group recently demonstrated the role of ZF encoded 3-OST-3 isoform in HSV-1 entry and spread [12]. In this context it is noteworthy that the human isoforms of 3-OST-2 enzyme is known to be utilized by HSV-1 for cell entry and spread [26]. In this study we evaluated the role of ZF encoded 3-OST-2 isoform in HSV-1 entry. As a first step ZF 3-OST-2 encoding open reading frame was successfully cloned into empty vector pCDNA3.1. The expression plasmid encoding ZF 3-OST-2 was then used in parallel with empty vector in a series of experiment using CHO-K1 cells which lack endogenous 3-OST enzymes and gD receptor. Our findings demonstrate that 3-OS HS generated by ZF encoded 3-OST-2 isoform mediates HSV-1 entry and cell to cell fusion in a manner similar to its human counterpart [26]. These results extend the growing list of the role of 3-OSTs in HSV-1 pathogenesis.

Previous studies with *in situ* hybridization in ZF have shown localized expression of 3-OST-2 in temporal and spatial regions of the brain [18]. In the context of 3-OST-2 being exclusively expressed in ZF brain along with the fact that HSV-1 is a neurotropic virus, opens one interesting possibility of HSV-1 usage of 3-OST-2 receptor during neuronal invasion in ZF model. Therefore, future studies on HSV-1 infection with ZF 3-OST-2 knock-outs will be interesting to rule out such possibilities. In ZF system differences in sulfated patterns of HS chain during embryonic development has also been suggested [27] which could affect 3-OST-2 expression and subsequent HSV-1 mediated damages in ZF model.

Future understanding on HSV-1 entry via specific cell receptor 3-OST-2 in ZF model can assists us in following ways. First it can provide the answer if receptor specificity is involved in HSV tropism especially in the brain. If true, strategies with small molecule or HS mimics can be developed to block 3-OST-2 dependent infections. Because 3-OST-2 is predominately expressed in brain regions in both human and ZF it is interesting to study antagonist to 3-OST-2 receptor preventing HSV-1 spread in neuronal regions. In this context our phage display generated 12-mer anti-HS and anti-3-OS HS peptides isolated against human 3-OST isoform will be very useful to test in cell culture model that exclusively expresses 3-OST-2 isoform and in intact ZF embryo model. Because ZF embryo is transparent it offers a unique opportunity to use real-time imaging using GFP-tagged-HSV-1 for receptor-specific localized replication or spread. In addition, ZF embryos are also an excellent organism to study inflammation process [8, 10] therefore it is also possible to delineate the involvement of immune mediators in the presence and the absence of 3-OST-2 receptor during HSV-1 infection. In summary, our work provides the initial platform for future investigations to study role of 3-OST-2 receptor in ZF model.

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HIGHLIGHTS

- Zebrafish 3-OST-2 isoform was necessary for the HSV-1 entry
- HSV-1 entry was strain independent.
- The results show 3-OST-2 role in HSV-1 entry and spread.

Figure 1. Cloning of zebrafish (ZF) encoded 3-OST-2 isoform

Zebrafish encoding 3-OST-2 plasmid was constructed by inserting the open reading frame of 3-OST-2 into pCDNA3.1 and the construct was designated pCDNA3.1-ZF-3-OST-2. The inserted sequence of 1100 bp of 3-OST-2 was verified after digestion using BamH1 and Xho1.

Figure 2. Expression of ZF 3-OST-2 in wild-type CHO-K1 cells results in HSV-1 entry (A). Dose response curve of HSV-1 entry into ZF expressing 3-OST-2 CHO-K1 cells. Resistant wild-type CHO-K1 cells were transfected with ZF-3-OST-2 at 2.5 μ g DNA resulted HSV-1 gL86 entry, similar to human 3-OST-3 expression. Cells transfected with empty vector pCDNA3.1 and human 3-OST-1 at 2.5 μ g DNA was used as a negative control. Cultured cells were plated in 96-well plates and inoculated with two-fold serial dilutions of β -galactosidase-expressing recombinant virus HSV-1 (KOS) gL86 at the plaque forming units (PFU) indicated. After 6 hr, the cells were washed, permeabilized and incubated with ONPG substrate (3.0 mg/ml) for quantitation of β -galactosidase activity expressed from the input viral genome. The enzymatic activity was measured at an optical density of 410 nm (OD₄₁₀). (B). HSV-1 entry into ZF 3-OST-2 expressing CHO-K1 cells was further confirmed by X-gal staining. Cells grown (4×10^6 cells) in six well dishes were challenged with β -galactosidase-expressing recombinant HSV-1 (gL86) at 15 pfu/cell. Wild-type CHO-K1 cells and human 3-OST-1 and 3-OST-3 expressing CHO-K1 cells were also infected in parallel as negative and positive control. After 6 h of infection at 37° C, cells

were washed with PBS, fixed and permeabilized, and incubated with X-gal (5 bromo-4 chloro-3-indoyl- β -D- galactosidase) at 1.0 mg/ml, which yields an insoluble blue product upon hydrolysis by β -galactosidase. Blue cells (representing viral entry) were seen as shown. Microscopy was performed using a 20 $\times$ objective of Zeiss Axiovert 100.

Figure 3.**Figure 3A. Clinical isolates of HSV-1 utilize ZF 3-OST-2 receptor during cell entry.**

CHO-IE β 8 cells transfected with expression plasmid of ZF 3-OST-2, human 3-OST-2 and pCDNA3.1 were seeded in a 96 well plate. The cells were then infected with various HSV-1 isolates at 40,000 PFU. Six hour post infection cells were washed with $1 \times$ PBS and the soluble substrate o-nitrophenyl- β -D-galactopyranoside (ONPG; ImmunoPure, Pierce) was added. The enzymatic activity was measured by a micro-plate reader (TECAN GENious Pro) at 405 nm.

Figure 3B. Co-expression of gD with ZF 3-OST-2 renders resistance to HSV-1 entry.

CHO-K1 cells were co-transfected with ZF 3-OST-2 expression plasmid and gD expression plasmid or pCDNA3.1. as indicated. The cells were then infected with reporter β -galactosidase expressing HSV-1 KOS gL86 virus at the doses indicated. After 6 h, cells were lysed and β -galactosidase activity was determined as a measure of virus entry by

adding o-nitrophenyl-b-D-galactopyranoside (ONPG) substrate. Viral entry was measured spectrophotometrically (OD₄₁₀). The assays were performed in triplicates and repeated four times with similar results. The mean values plus standard deviations from a representative experiment are shown.

Figure 4. Enzymatic removal of cell surface heparan sulfate (HS) by heparinase treatment in ZF 3-OST-2 expressing CHO-K1 cells reduces HSV-1 infection

The cultured cells expressing ZF 3-OST-2 were treated with heparinase II/III (1.5 U/ml; white bar) or mock treated (black bar) in the first well of the 96 well plate and followed by exposing cells to HSV-1 (KOS) gL86 at 10^4 PFU/well and viral entry was quantitated 6 hr later by ONPG assay (panel A) and X-gal assay (panel B). The heparinase treated ZF-3-OST-2 cells had significantly lesser number of blue cells (upper panel in panel B) compared to mock treated ZF-3-OST-2 cells (lower panel in panel B).

Figure 5. ZF 3-OST-2-expressing target CHO-K1 cells gain the ability to fuse with effector cells co-expressing HSV-1 glycoproteins gB, gD, gH, and gL while heparinase treatment significantly blocks ZF 3-OST-2 mediated fusion

The target CHO-K1 cells were transfected with plasmids expressing human (H) 3-OST-2 and ZF 3-OST-2 (as indicated), and luciferase reporter gene. The effector CHO-K1 cells were transfected with HSV-1 glycoproteins gB, gD, gH, and gL, and T7 RNA polymerase. CHO-K1 effector cells expressing control plasmid without HSV-1 glycoproteins were used as a negative control. In addition, target CHO-K1 cells expressing human or ZF 3-OST-2 were treated with heparinase II/III (1.5U/ml) or left untreated for 2 hr prior to co-cultivation with effector CHO-K1 cells expressing four HSV-1 essential glycoproteins (gB, gD, gH-gL; 0.5 µg DNA each glycoprotein). A luciferase reporter assay was performed 24 h after the two cell populations were mixed together. Cell fusion was measured in relative luciferase units (RLUs) using a Sirius luminometer (Berthold Detection System).