Initial evidence on differences among Enterovirus 71, Coxsackievirus A16 and Coxsackievirus B4 in binding to cell surface heparan sulphate

Hamid Reza Pourianfar · Kristin Kirk ·
Lara Grollo

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Abstract Cell surface heparan sulphate (HS) mediates infection for many viruses from diverse families. We demonstrated significant antiviral potencies for a number of HS mimetics against a cloned strain of Enterovirus 71 (EV71) in a previous study. Thus, the involvement of HS in mediating viral infection of isolates of human enteroviruses was investigated in Vero and human neural cells in the present work. In both cell lines, heparin and pentosan polysulphate significantly inhibited both infection and attachment of low passage clinical isolates of EV71 and Coxsackievirus A16 (CVA16) but showed no affect on Coxsackievirus B4 (CVB4) ($p < 0.05$). In addition, enzymatic removal of cell surface HS by heparinase I prevented binding of the clinical EV71 by nearly 50 % but failed to significantly inhibit CVA16 or CVB4 binding in Vero cells. Overall, the findings of this study provides evidence that whilst highly sulphated domains of HS serve as an essential attachment co-receptor for EV71, HS might be used as an alternative attachment receptor by the other member of Human Enterovirus group A, CVA16. In addition, HS may not mediate early infection in CVB4.

Keywords Human Enterovirus 71 · Heparan sulphate · Viral attachment · Virus receptor

Introduction

Cell surface heparan sulphate (HS) has long been known to have the capability to interact with many viruses of diverse families, and thus, to play key roles in mediating infection for these viruses [25]. The virus-HS interaction for many viruses originates from multiple passages of the virus, leading to adaptation to cell culture. This phenomenon has been reported for several viruses, including foot and mouth disease virus (FMDV) [1, 5, 12, 18], Sindbis virus (SIN) [9], and Venezuelan equine encephalitis virus (VEE) [2], where the virus adapted in cell culture to bind to multiple receptors. This being said, the use of cellular HS by viruses might not be totally attributed to adaptation to cell culture, which can often be confirmed by using low passage clinical strains for which cell surface HS may serve as an attachment co-receptor. In fact, following sialic acid, HS is considered the second largest group of carbohydrate receptors for human viruses of various families, such as *Adenoviridae*, *Flaviviridae*, *Herpesviridae*, *Papillomaviridae* and *Paramyxoviridae* [14].

The possible role of cell surface HS in mediating infection of Human Enteroviruses (HEVs) has mostly been reported for serotypes of HEV group B, including Echovirus [8], Coxsackievirus B3 (CVB3) [24], swine vesicular disease virus (SVDV) [6], and Coxsackievirus A9 (CVA9) [11]. Recently, we demonstrated that several soluble HS mimetic compounds, including heparin (Hep), pentosan polysulphate (PPS) and HS, exhibited potent antiviral activities against a cloned strain of EV71 through interfering with the viral attachment in Vero cells [16],

H. R. Pourianfar
Iranian Academic Centre for Education, Culture and Research
(ACECR)-Mashhad Branch, P.O. Box 91775-1376, Mashhad,
Iran

H. R. Pourianfar · K. Kirk · L. Grollo (✉)
Environment and Biotechnology Centre, Swinburne University
of Technology, Hawthorn, VIC 3122, Australia
e-mail: laragrollo@gmail.com

suggesting a role for cellular HS in mediating early move of the EV71 infection. Later, it was proved by other group that EV71 uses heparan sulphate as an attachment receptor in RD cells [20]. Therefore, we extended our studies in order to investigate whether cellular HS may mediate the early infection of clinical isolates of CVB4 (as a member of HEV-B) and CVA16 (as a member of HEV-A) for which the role of HS has not yet been reported. The HS usage by a low passage clinical isolate of EV71 was also compared to that of a cloned strain of EV71 in order to determine the contribution of cell culture adaptation to the previously reported virus-HS interaction. In addition to Vero cells as the main cell line in this study, we tested interaction of the Enteroviruses with cellular HS in a human neural cell line.

Materials and methods

Cell lines and viruses

Vero (provided by Prof. Peter C. McMinn (Central Clinical School, University of Sydney, Australia) and human neural cells (SK-N-SH, Australia Cell, Catalogue number 86012802) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Mulgrave, VIC, Australia) with high glucose supplemented with 10 % heat inactivated fetal bovine serum (FBS, Invitrogen, Mulgrave, VIC, Australia). The cells were not passaged more than 20 times after reviving from the original stocks. Vero and neural cells were seeded at 1.5×10^4 and 1.35×10^4 cells/well, respectively in 96-well plates (Becton–Dickinson, North Ryde, NSW, Australia) followed by incubation at 37 °C/5 % CO₂ until 80 % confluent.

A cloned isolate of EV71 strain 6F/AUS/6/99 (GenBank Accession number DQ381846) was supplied by Prof. Peter C. McMinn. Low passage clinical isolates of the following human Enteroviruses were used: EV71 (isolate number 99018233); Coxsackievirus A16 (isolate number CAIG 9902-2745-4PMEK); and Coxsackievirus B4 (isolate number 99039838), which were supplied by Dr. Jullian Druce of the Victorian Infectious Disease Reference Laboratories (VIDRL, North Melbourne, VIC, Australia). The virus titers were determined in Vero cells as described previously [15]. The clinical viral isolates were kept low passage (<5 times) in order to eliminate the possibility of developing HS binding virus mutants through adaptation to cell culture. The final viral supernatants were aliquoted and stored at −80 °C until use.

Reagents

Pentosan polysulphate sodium salt (PPS) (provided by Dr. John McEwan, Sylvan Pharmaceuticals Pty Ltd, Bondi

Junction, NSW, Australia) and heparin sodium salt (Hep) from bovine intestinal mucosa (≥ 140 USP units/mg, catalogue number H0777) purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) were dissolved in sterile distilled water (10 mg/mL) and stored at 4 °C until use, as described previously [16].

Heparinase buffer was prepared by supplementing PBS with 4 mM CaCl₂, 0.5 mM MgCl₂, 0.1 % (W/V) glucose, 1 % (V/V) FBS, and 0.5 % (W/V) BSA with a pH adjusted to 7 [24]. The buffer was aliquoted and stored at 4 °C before use within 1 week of preparation. Heparinase enzymes I (Catalogue number H2519) and III (Catalogue number H8891) were reconstituted in the heparinase buffer at 295 Sigma U/mL and 300 Sigma U/mL, respectively. The enzyme solutions were aliquoted and stored at −20 °C until use.

Infectivity assay with soluble glycosaminoglycans

The antiviral effect of the glycosaminoglycans (GAGs) mimetics was measured as described previously [16]. In short, 80 % confluent cells were infected with 50 μ L/well of virus at 100 TCID₅₀, followed by the addition of 100 μ L/well of Hep or PPS at various concentrations (ranging from 3.9 to 250 μ g/mL) in triplicates. After incubation at 37 °C/5 % CO₂ for a further 2 days, the results were quantified using 20 μ L/well of MTT reagent (Invitrogen, Mulgrave, VIC, Australia). The plates were then incubated in a humidified 37 °C incubator with 5 % CO₂ for 2–3 h after which the formazon was dissolved with DMSO (50 μ L/well) followed by another 10-min incubation. The colour change was then recorded using a microplate reader at 540 nm. The virus inhibition percentages were measured using the following equation: $T - V_c / C_c - V_c$, where T = the optical density (OD) of compound-treated cells, V_c = OD of virus control, C_c = OD of cell control [19]. The antiviral activity curve was then generated by plotting virus inhibition percentages against compound concentrations.

Virus binding assay

The effect of the compounds on virus attachment was assessed by a virus binding assay, modified from De Logu et al. [3] with minor changes. Briefly, 80 % confluent cells were pre-chilled at 4 °C for 1.5 h followed by infection with the virus (at 100 TCID₅₀) which had been pre-incubated with the compound (125 μ g/mL) at 4 °C for 45 min [2]. The GAGs were given an opportunity to inhibit viral binding at 4 °C for 2 h; afterward the monolayers were washed three times with PBS. 150 μ L of DMEM/5 % FBS was then added to each well and plates were incubated for a further 2 days at 37 °C/5 % CO₂. Quantification of results was performed as described above.

GAG-lytic enzyme treatment

To determine the use of cell surface HS as a receptor, cells were treated with GAG-lytic enzymes prior to infection with virus, as previously depicted [7, 10, 21, 24]. Briefly, 80 % confluent Vero cells were washed three times with PBS, the cells were then treated with 10 U (35 μ L/well) of heparinase I or heparinase III. Virus and cell control well were added, where cells received 35 μ L of heparinase buffer containing no enzymes. The plates were then incubated at 37 °C/5 % CO₂ for 60 min, followed by removing supernatant and washing the monolayers three times with warm PBS. Then, 50 μ L of DMEM/5 % FBS was added to each well and plates were pre-chilled at 4 °C for 90 min, afterward 100 TCID₅₀ of virus was added to all the wells followed by incubation at 4 °C for 2 h. After washing three times with PBS to eliminate un-bound viruses, the wells were overlaid with 150 mL of the growth medium (DMEM + 5 % FBS) followed by incubation at 37 °C/5 % CO₂ for 48 h. The effect of GAG-lytic enzymes on virus attachment was determined using MTT staining as described above.

Statistical analysis

All the treatments were applied in triplicate, and each experiment was independently repeated at least two times. An unpaired independent two-sample *t* test was used to compare means with each other, with a significance level (*p* value) of 5 % or 1 %.

Results

Anti-enteroviral activity of Hep and PPS

As a general trend, 125 μ g/mL was the lowest cytotoxic concentration at which both compounds exerted viral inhibition greater than 40 % against the clinical and cloned isolates of EV71 and greater than 30 % against CVA16 in Vero cells. Antiviral activities of Hep and PPS against the cloned EV71 strain at most of the concentrations tested were greater than those against both the clinical strains of EV71 or CAV16 (*p* < 0.05) (Fig. 1). In addition, there was no statistically significant difference in virus inhibition between Hep and PPS against the clinical strains of EV71 or CVA16 (*p* > 0.05). Likewise, the antiviral response curves of Hep or PPS against the clinical strain of EV71 did not significantly differ from those against the clinical strain of CVA16 (*p* > 0.05).

None of the tested concentrations of Hep (Fig. 1b) or PPS (Fig. 1a) could significantly prevent viral infection of the clinical strain of CVB4 in Vero cells, aside from the concentration 15.62 μ g/mL at which PPS exerted an antiviral activity of 45 % that was significantly different from the other concentrations (*p* < 0.05) (Fig. 1a).

While exhibited no cytotoxicity, Hep and PPS exerted substantial antiviral potencies against the infection of clinical EV71 and CVA16 in neural cells, which were also much more pronounced than those in Vero cells (*p* < 0.05). However, no significant viral prevention was observed with the cloned EV71 or CVB4 (data not shown).

Fig. 1 Anti-enteroviral activities of GAGs in Vero cells. 80 % confluent Vero cells infected with 100 TCID₅₀ of the cloned Enterovirus 71, clinical Enterovirus 71, clinical Coxsackievirus A16, or clinical Coxsackievirus B4 strains were exposed to PPS (a) or Hep (b) at seven different concentrations ranging from 3.9 to 250 μ g/mL at which no significant cytotoxicity had been observed in

the previous study [16]. The values represent mean $\pm$ SD virus inhibition percentage collected at 48 h post infection from at least three independent experiments. The results of antiviral actions of Hep and PPS against the cloned EV71 in Vero cells have been reproduced from the previous study [16]

Enteroviral attachment in the presence of GAGs compounds

In order to assess the possible interaction of cell surface HS with the Enteroviruses, the effects of the soluble GAGs (at 125 µg/mL) on preventing Enteroviral attachment was evaluated. The results revealed that the cloned EV71, clinical EV71 and clinical CVA16 isolates were considerably prevented from binding to Vero cells by Hep and PPS. While Hep could prevent attachment of the clinical strain of EV71 by more than 68 %, the figures were over 64 % for the cloned EV71 and 43 % for the clinical CVA16. However, there was no statistically significant difference in virus binding inhibition by Hep among the three viruses ($p > 0.05$) (Fig. 2).

PPS inhibited the binding of the cloned EV71 and clinical EV71 strains by about 50 % and over 65 %, respectively (Fig. 2). Nevertheless, it exerted a moderate inhibition (42 %) for attachment of CVA16 to Vero cells, making a statistical difference in preventing virus attachment by PPS between clinical EV71 and clinical CVA16 ($p < 0.05$). CVB4 attachment was not seen to be significantly suspended by Hep or PPS (Fig. 2), causing a very significant difference in viral binding inhibition between CVB4 and the HEV-A isolates ($p < 0.01$).

Fig. 2 The effect of GAGs on Enteroviral binding to Vero cells. Pre-chilled Vero cells were treated with the mix of Hep or PPS (each at 125 µg/mL) and virus that had been pre-incubated together at 4 °C for 45 min. Viral attachment was then allowed to occur at 4 °C. The values represent mean $\pm$ SD of virus binding inhibition percentage collected at 48 h post infection from at least two independent experiments

In neural cells, the effects of Hep and PPS on the HEV-A isolates were much more pronounced than those in Vero cells. However, again the compounds could not efficiently prevented CVB4 binding neural cells.

Controlled, repeated microscopic observations also demonstrated that observed viral CPE inhibited by Hep or PPS during the binding assay was in accordance with the MTT-quantified results of the attachment assay (Fig. 3).

Enteroviral attachment in the absence of cell surface HS

In order to further confirm the role of HS in interacting with virus adsorption, the reduction of viral CPE following treatment with heparinases I and III was investigated, as stated elsewhere [24]. Vero cells were treated with heparinase I or heparinase III, after which attachment was performed at 4 °C as described for virus binding assay. While treatment with heparinase I inhibited the cloned EV71 attachment to Vero cells by over 25 %, it demonstrated viral attachment inhibition of almost 50 % for the clinical EV71, making a statistically significant difference ($p < 0.05$) between these isolates of EV71. Treatment of Vero cells with heparinase III did not demonstrate a considerable viral inhibition for the cloned EV71 or clinical EV71 strains, as opposite to heparinase I ($p < 0.05$). In addition, no meaningful viral inhibition was observed with the enzyme-treated cells infected with CVA16 or CVB4, as compared to those infected with the cloned or clinical isolates of EV71 ($p < 0.05$) (Fig. 4).

The statistical analyses also revealed that there was a significant difference between viral attachment prevented by Hep or PPS and that of observed in the heparinase-treated cells ($p < 0.05$) for cloned EV71 and clinical CVA16, but not for the clinical EV71 isolate ($p > 0.05$).

The results of heparinase treatment did not show significant virus inhibition for any of the viruses in neural cells.

Discussion

The outstanding inhibition of clinical EV71 attachment by the soluble GAGs suggested that both Hep and PPS could significantly interfere with the viral binding to Vero cells. In line with these findings, removing the cell surface HS by heparinase I obviously reduced the clinical EV71 attachment to Vero cells, and this inhibition was significantly greater in comparison with the cloned EV71 ($p < 0.05$). The cellular HS molecule itself consists of three segregated blocks, including GlcA-(1,4)-GlcNAc disaccharides (*N*-acetylated (NA)), highly sulphated IdoA-(1,4)-GlcNS disaccharides (*N*-sulphated (NS)), and transition domains

Fig. 3 Micrographs of inhibition of Enteroviral binding to Vero cells. Inhibition of virus-induced CPE during the attachment assays was photographed through an inverted microscope (1,000 times magnified, Olympus, CKX 41, Japan) before washing the cells with PBS. The micrographs were recorded for every well of the all independent replicates along with cell and virus controls. The panel of cell control represents the average cell control observed for all the assays. The conditions of virus binding assay were the same as described in Fig. 2

Fig. 4 Relative effect of heparinase enzymes on Enteroviral binding to Vero cells. Vero cells were treated with the heparinase enzymes at 10 U (35 μ L/well). Then, the cells were pre-chilled at 4 °C followed by infection with virus. Viral attachment was allowed to occur at 4 °C. The values represent mean $\pm$ SD virus binding inhibition percentage collected at 48 h post infection from at least two independent experiments

[17]. Accordingly, heparinase I primarily cleaves NS domains and to a lesser extent catalyzes NA domains; while heparinase III acts exclusively on NA domains of the cell surface HS [4]. Hence, it can be suggested that highly sulphated, Hep-like domains of cell surface HS may serve as a specific and necessary co-receptor for binding of the clinical isolate of EV71 to Vero cells.

The EV71 isolate used in this study was a very low passage clinical EV71 and was also kept low passage (<5). In addition, the cells were not passaged more than 20 times. Therefore, the HS-virus interaction seen in this study is less likely to be originated from adaptation to cell culture. It can be thus proposed that cellular HS might mediate an early move for binding of the clinical isolate of EV71 to Vero cells, which could further assist recognition of other receptors, as shown for other HEV members such as SVDV [6]. These results can be also supported with the recent findings that a strain of EV71 was shown to use HS as an attachment receptor in RD cells [20].

It is noteworthy that although Human Scavenger Receptor Class B, Member 2 (SCARB2) was previously demonstrated to be the dominant EV71 receptor in epithelial cell lines [22], the presence of another minor receptor(s) in one of the tested susceptible cell lines was also suggested, which could facilitate EV71 infection through a SCARB2-independent pathway [22]. This is also in agreement with a general fact that picornaviruses

easily adapt to bind to different co-receptor(s) even in the same cell type due to having the flexibility in receptor usage [1, 6, 24]. Moreover, Vero cells, as an epithelial cell line, do not support EV71 replication through Human P-selectin Glycoprotein Ligand-1 (PSGL-1) that was reported to serve as an EV71 receptor primarily in leukocytes [13]. Thus, we would propose that the infection of Vero cells by the clinical isolate of EV71 could be mediated or facilitated by HS as an alternative attachment co-receptor in addition to or independent of SCARB2.

Unlike the clinical isolate of EV71, heparinase treatment did not prevent the cloned EV71 attachment as much as it was expected from the observed antiviral activities of soluble GAGs against the virus ($p < 0.05$). One explanation might be that this highly passaged cloned strain of EV71 might have lost its ability to interact with HS through consecutive passages. It can also be suggested that this strain of EV71 may largely utilize one of the two recently suggested candidates for cell receptors of EV71 for fibroblasts and epithelial cell lines; SCARB2 [22], or sialic acid-linked glycans [23], independent of cell surface HS. Therefore, whilst the role of HS in interacting with this cloned strain of EV71 may not be completely overlooked, the virus adsorption is not essentially and selectively dependent on cell surface HS in Vero cells. The structural protein genes of the clinical and cloned strains of EV71 could be examined in further studies to confirm if there are motifs in viral capsid proteins that have conferred the HS usage capability to the clinical isolate virus, in comparison with the cloned strain.

Despite the observed significant prevention of CVA16 infection and attachment by the HS mimetics, no considerable viral inhibition for this virus was observed in cells treated with the heparinase enzymes. Therefore, it cannot be confirmed that cell surface HS serves as a selective and necessary attachment co-receptor for CVA16 adsorption. It has recently been discovered that SCARB2 can serve as an attachment receptor for CVA16 in epithelial cell lines [22]. Thus, one could propose that the clinical CVA16 might use SCARB2 or another yet to be identified cell receptor to bind to Vero cells, independent of HS. This being said, the possible role of HS in assisting early infection or binding of CVA16 to Vero cells cannot be completely ruled out, as interaction of this very low passage clinical isolate with the HS mimetics was clearly seen in this study.

The trend of infection caused by CVB4 was considerably different when compared to EV71 and CVA16. Overall, no significant inhibition by the GAGs or heparinase treatment was observed against CVB4 infection or attachment. Bearing in mind that CVB4 is a member of HEV-B, these findings appear to be in agreement with previous studies, where it was observed that Hep failed to

inhibit infection of virus strains of HEV-B, including Cocksackievirus B2 and B3 in RD cells [8] and several isolates of CVA9 [11]. However, those contrast with what was reported for several isolates of Echoviruses [8, 11], two isolates of CVA9 [11] and a laboratory strain of CVB3 [24] as other members of HEV-B that were shown to utilize HS as an attachment receptor. With regard to CVB3 [24], it was not confirmed whether the binding of CVB3 to HS might be a cell culture phenomenon or a characteristic of the cell receptors of CVB3 in nature, as a clinical isolate of CVB3 was not used. Thus, it may not be possible to generalize the HS non-binding CVB4 studied by us to all the members of HEV-B.

Recently a single amino acid change at position 132 in VP1 was demonstrated to confer the capability of HS interaction to two isolates of CAV9 and several other types of HEV-B [11]. However, it was further clarified that this HS usage phenomenon was rare in CVA9 isolates, as none of 15 other CVA9 isolates were inhibited by Hep as a HS mimetic compound [11]. It was therefore concluded that the HS usage in CVA9 and some other members of HEV-B might have been arisen from a cell culture adaptation, implying the potential flexibility of receptor usage by Enteroviruses [11]. Therefore, it could be concluded that sequence analyses, HS mimetics studies and the history of virus cultures all would be important to elucidate possible interaction of cell surface HS with Enteroviruses (belonging to either HEV-A or HEV-B).

In this study, the importance of cell surface HS in interacting with the Enterovirus isolates was also examined in human neural cells. While no viral prevention was observed against infection and attachment of the cloned EV71 and CVB4 in neural cells, significant antiviral potencies against infection and binding of the clinical isolates of EV71 and CVA16 in neural cells was demonstrated, which initially suggested a role for HS in assisting early infection or binding of these viruses. However, heparinase treatment of neural cells failed to prevent viral attachment of the viruses. Thus, binding of the HEV-A or -B isolates to neural cells may not be necessarily dependent on the presence of HS. In the absence of any confirmed neural specific receptor for EV71 or CVA16, this issue warrants further investigations. In addition, although the involvement of cellular HS in mediating Enteroviral infection could not be confirmed in neural cells, the strong antiviral actions of Hep and PPS against the infection of clinical EV71 and CVA16 in these cells might have clinical implications.

As opposite to CVA16 and CVB4, cell surface HS was clearly demonstrated to interact with a low passage clinical isolate of EV71 in binding to Vero cells. Given the recent findings on HS usage by EV71 in RD cells [20], it could be proposed that cellular HS plays an important role in early

move of EV71 infection in epithelial host cells. Even though the role of HS in mediating the viral infection of CVA16, other member of HEV-A, was not proved to be crucial, the observed antiviral potencies of the HS mimetics against the CVA16 infection (in both Vero and human neural cells) may have potential for further studies on the treatment of the virus.

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