Short Communication

Resistance to pseudorabies virus infection in transformed cell lines expressing a soluble form of porcine herpesvirus entry mediator C

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Received 2 July 2003
Accepted 1 October 2003

Porcine herpesvirus entry mediator C (HveC) is an alphaherpesvirus receptor that binds to virion glycoprotein D (gD). Porcine HveC mediates entry of pseudorabies virus (PRV), herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and bovine herpesvirus type 1 (BHV-1). In order to assess the antiviral potential of a soluble form of porcine HveC, Vero cells were transformed with the chimeric gene expressing a fusion protein (PHveCIg) consisting of an extracellular domain of porcine HveC and the Fc portion of human IgG1. The transformed cell lines expressing PHveCIg showed marked resistance to PRV infection. Resistance to infection by other alphaherpesviruses (HSV-1 and BHV-1) was also observed in the transformed cell line. The present results demonstrate that a soluble form of porcine HveC is able to exert a significant antiviral effect against pseudorabies virus and other alphaherpesvirus infection in vitro.

Pseudorabies virus (PRV), genus Varicellovirus, subfamily Alphaherpesvirinae (Kit, 1994), causes severe disease in piglets and leads to latent infection in all surviving pigs. Members of the alphaherpesvirus subfamily, including PRV, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and bovine herpesvirus type 1 (BHV-1) as representative members, are neurotropic, have a short replicative cycle and have a broad host-range. Infection in the natural host is characterized by lesions in the epidermis, usually on a mucosal surface, with spread of virus to the nervous system and establishment of latent infections in neurons.

Interaction between alphaherpesvirus envelope glycoprotein gD and a specific cellular receptor is required for virus entry into mammalian cells (Campadelli-Fiume et al., 1988; Spear et al., 2000). Binding of alphaherpesviruses to cells occurs primarily through an interaction of virion glycoprotein gC with cell-surface heparan sulfate, whereas fusion between the virion envelope and cell membrane requires glycoproteins gB, gD, gH and gL. To date, five human alphaherpesvirus receptors have been identified: herpesvirus entry mediator (HVEM, also known as HveA and TNFRSF14), a TNF receptor-related protein (Montgomery et al., 1996); three immunoglobulin superfamily members, HveB (PRR2, nectin-2), HveC (PRR1, nectin-1), and HveD (PVR, CD155); and 3-O-sulfated heparan sulfate (Cocchi et al., 1998; Geraghty et al., 1998; Warner et al., 1998; Shukla et al., 1999). Several lines of evidence suggest that alphaherpesvirus gD interacts with a cell-surface receptor in addition to heparan sulfate to mediate virus entry and that, in certain cell types, PRV, HSV-1 and BHV-1 can use a common gD receptor for entry (Campadelli-Fiume et al., 1988; Spear, 1993).

The nectin family consists of proteins related in sequence and structure to poliovirus receptor (nectin-2/HveB, nectin-1/ HveC and nectin-3). They have the same immunoglobulin-like domain structures (one V domain followed by two C2 domains) (Mendelsohn et al., 1989; Eberle et al., 1995; Lopez et al., 1995; Reymond et al., 2000; Satoh-Horikawa et al., 2000). Human, mouse and porcine nectin-1 have the broadest specificity for mediating alphaherpesvirus entry and are highly conserved in primary sequence. It seems likely that various mammalian forms of nectin-1 will prove to be pan-alphaherpesvirus entry receptors. Nectin-1 and nectin-2 appear to be expressed by a broad range of tissues and cells (Cocchi et al., 1998; Geraghty et al., 1998; Warner et al., 1998). Some isotypes of nectin-1, nectin-2 and nectin-3 can localize to cadherin-based adherens junctions in epithelial cells through binding of their cytoplasmic tails to PDZ domains in afadin, a cytoplasmic protein that binds to actin filaments and components of cadherin-based
cell junctions (Mandai et al., 1997; Asakura et al., 1999; Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000).

To construct plasmids expressing soluble forms of porcine HveC (PHveCIg) or murine HveA (MHveAIg), pCXN2 expression vectors containing chimeric genes that encode fusion proteins consisting of an extracellular domain of porcine HveC or murine HveA and the Fc portion of human IgG1 were constructed. The cDNA of the extracellular domain of porcine HveC was amplified as described by Milne et al. (2001). The extracellular domain of murine HveA cDNA was amplified by using RT-PCR, in which an mRNA isolated from concanavalin A (Con A)-stimulated splenocytes obtained from BALB/c mice was used as a template. The PCR primers used to amplify the cDNA were 5'-TAACTCGAGCTCTTGGCCTGAAGTTTC-3' and 5'-TTAAGGATCCGAGGAGCAGGTGGTGTCT-3'. Each cDNA was inserted into the XhoI and BamHI sites of a plasmid carrying IgG1-Fc DNA (Nakagawa et al., 1998), and the sequence verified. Each plasmid was digested with XbaI and end-filled with Klenow fragments, and then a SalI linker was inserted into the blunt-ended site. Then, the plasmids were digested with XhoI and SalI to obtain the chimeric gene fragments. Each fragment was inserted into an XhoI site of pCXN2 vector (Niwa et al., 1991). To detect MHveAlg, polyclonal antibodies against MHveAlg were raised in a New Zealand White rabbit immunized with the purified MHveAlg. Vero cells were transfected with each plasmid and stable transformants were selected as described previously (Ono et al., 1995).

PHveCIg and MHveAlg were expressed under the control of the CAG promoter (cytomegalovirus IE enhancer and chicken β-actin promoter; Fig. 1A) in the transformed cell lines. A total of 35 G418-resistant cells were cloned from different transfection experiments and subsequently grown. A rabbit anti-human HveC antibody-specific band (Krummenacher et al., 1998) was detected by Western blot analysis in 15 of the 35 cell lines. All of the PHveCIg-expressing cell lines exhibited resistance to PRV infection, indicating an obvious correlation between the resistance to PRV infection and expression of PHveCIg. To examine the resistance to PRV infection in the transformed cell

![Fig. 1.](image)
The size of plaques was measured by using an inverted microscope with an ocular containing a 20x magnification. Although more than 100 plaques appeared, exact numbers were not countable.

Detection of PHveCIg was performed by Western blot analysis. In view of the possibility of Fc receptor-mediated binding of the protein to virions having an effect on entry, cell lines expressing MHveAIg were established, and two MHveAIg-expressing cell lines were chosen as control cell lines (Fig. 1B).

To compare resistance to PRV infection, the transformed cell lines were infected with PRV strain YS-81. The cells were cultured for 24 h and washed twice with DMEM, and then maintained in 1 ml of DMEM for further 24 h to accumulate PHveCIg or MHveAIg in the medium. The following day, 50 or 500 p.f.u. of PRV was added to the medium to adsorb onto the cells. Numbers of plaques in the PHveCIg-expressing cell lines (C-A5, C-A6 and C-B1) were extensively suppressed as compared with the non-expressing cell line (C-C2), the MHveAIg-expressing cell lines (A-B1 and A-C2) and Vero cells 2 days post-infection (Table 1). The plaque assays demonstrate that all of the transformed cell lines expressing PHveCIg showed remarkable resistance to PRV infection. In contrast, non-expressing cell line C-C2 (in which the presence of the DNA specifying the PHveCIg gene was confirmed by Southern blot analysis) and the MHveAIg-expressing cell lines were as susceptible to PRV infection as Vero cells.

When the PHveCIg-expressing cell lines were inoculated with 500 p.f.u. of PRV, several smaller plaques were detected. The size was definitely smaller than those observed in the control cell lines (Table 1). To monitor virus growth in the C-A6 cell line and Vero cells, virus infectivities in the media were titrated. Infection of the C-A6 cells with 0·01 p.f.u. per cell yields 10000-fold less infectious virus in comparison to Vero cells (data not shown). These results indicate several possible effects of PHveCIg on the suppression of virus growth. First, PHveCIg inhibits initial infection of C-A6 cells. Second, PHveCIg also inhibits secondary infection of C-A6 cells by free virus released in the first round infection. Third, PHveCIg inhibits secondary infection in C-A6 cells mediated by cell-to-cell spread. Fourth, PHveCIg neutralizes virus produced in the C-A6 cells, resulting in inhibition of infection of Vero cells.

The C-A6 cell line and one of the transformed cell lines expressing MHveAIg, the A-B1 cell line, were chosen for further examination of resistance to infection by other alphaherpesviruses. These cell lines were infected with 50 p.f.u. of HSV-1 strain VR-3 or BHV-1 strain LA as described above. In the C-A6 cell line expressing PHveCIg, numbers of plaques were extensively suppressed as compared with Vero cells 3 days post-infection (Fig. 1D). In control experiments, the A-B1 cell line expressing MHveAIg was protected from HSV-1 infection but not from BHV-1 infection as expected, since HveA acts as a gD receptor for HSV-1, but not for BHV-1 (Spear et al., 2000). However, the C-A6 cell line failed to show any protection against vaccinia virus strain WR infection compared with control cell lines (Fig. 2A). These results demonstrate that the porcine HveC-domain of PHveCIg expressed from the transformed cell line specifically inhibits infection with the alphaherpesviruses.

To assess the role of secreted PHveCIg in resistance to PRV infection, supernatant from the C-A6 cell line was collected

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expression of PHveCIg</th>
<th>Expression of MHveAIg</th>
<th>Plaque number(\dagger)</th>
<th>Plaque size (mm)(\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 p.f.u. per dish</td>
<td>500 p.f.u. per dish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-A5</td>
<td>+</td>
<td>–</td>
<td>0</td>
<td>0·31 ± 0·01</td>
</tr>
<tr>
<td>C-A6</td>
<td>+</td>
<td>–</td>
<td>0</td>
<td>0·27 ± 0·03</td>
</tr>
<tr>
<td>C-B1</td>
<td>+</td>
<td>–</td>
<td>0</td>
<td>0·35 ± 0·04</td>
</tr>
<tr>
<td>C-C2</td>
<td>–</td>
<td>–</td>
<td>53·5 ± 6·4</td>
<td>&gt;100(\dagger)</td>
</tr>
<tr>
<td>A-B1</td>
<td>–</td>
<td>+</td>
<td>55·5 ± 3·4</td>
<td>&gt;100(\dagger)</td>
</tr>
<tr>
<td>A-C2</td>
<td>–</td>
<td>+</td>
<td>48·5 ± 3·2</td>
<td>&gt;100(\dagger)</td>
</tr>
<tr>
<td>Vero</td>
<td>–</td>
<td>–</td>
<td>50·5 ± 9·2</td>
<td>&gt;100(\dagger)</td>
</tr>
</tbody>
</table>

\*Detection of PHveCIg was performed by Western blot analysis.
\*Detection of MHveAIg was performed by Western blot analysis.
\*The values are the means of at least triplicated infections and standard deviations are shown.
\*Although more than 100 plaques appeared, exact numbers were not countable.
\*The size of plaques was measured by using an inverted microscope with an ocular containing a micrometer. The values are the means of a total of ten plaques and standard deviations are shown.
at 20 min intervals and incubated with PRV for 1 h. Each sample containing viruses was adsorbed to Vero cells. In a virus plaque reduction assay (Fig. 2A), it was found that C-A6 cell supernatant protected Vero cells against PRV infection. In contrast, A-B1 cell supernatant failed to protect against PRV infection. Protein G-Sepharose 4B was added to the culture supernatant to capture the secreted fusion protein and analysed by Western blotting, which confirmed that each fusion protein was secreted into the supernatant (Fig. 2A).

Since the antiviral potential of secreted PHveCIg in 1 h-cultured supernatant was not enough to show complete protection (Fig. 2A), the role of cell-bound PHveCIg in resistance to PRV infection was examined. C-A6 cells were washed twice with DMEM just before virus inoculation and infected with 0–1 p.f.u. per cell of PRV. PRV entry 3 h post-infection was examined by indirect immunofluorescence assay using anti-EP0 antibodies. The lower parts show magnification of each panel (×40).

Consistent with the earlier studies (Geraghty et al., 1998; Krummenacher et al., 1998), a secreted form of HveC, PHveCIg, blocked infection of HSV-1 in addition to PRV and BHV-1. It is likely that the resistance to alphaherpesvirus infection is mediated at least in part by inhibition of the interaction between gD and simian HveC by the secreted PHveCIg. In the present study, cell-bound PHveCIg is also concerned with the inhibition of PRV infection, in addition...
to the secreted PHveCIg. Although the mechanism by which cell-bound PHveCIg inhibits alphaherpesvirus infection is unclear, it is likely that PHveCIg sterically interferes with the early assembly of a fusion complex involving multiple components from the alphaherpesvirus envelope and cell plasma membrane. Consequently, the transformed cell lines exhibit the resistance to alphaherpesvirus infection.

In the present study, transformed cell lines expressing PHveCIg showed significant resistance to PRV infection. This resistance was much more striking than that observed in PRV-resistant transformed cell lines expressing transgenes repressing PRV immediate-early gene transcription (Ono et al., 1995, 1998; Tasaki et al., 2001). It is noteworthy that PRV entry into the C-A6 cells was almost completely inhibited (Fig. 2B). The demonstration that PHveCIg expressed in the transformed cell lines is sufficient to compete with PRV infectivity may provide a basis for the future development of agricultural livestock for enhanced disease resistance to pseudorabies.

ACKNOWLEDGEMENTS

This work was supported by Grant-in-Aid for Scientific Research (B)(2) from The Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Drs G. H. Cohen and R. J. Eisenberg for anti-human HveC rabbit serum R154, Dr R. S. B. Milne for pRM361 expressing porcine HveC, Dr J. Miyazaki for pCXN2, Dr T. Suzutani for HSV-1 strain VR-3, and Dr S. Katayama for monoclonal antibody against PRV gD.

REFERENCES


