Development of a Method That Delivers Drugs to Enveloped Viruses

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Hepatitis C virus (HCV) infection leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in 50–80% of the cases. Interferons (IFNs) and the nucleoside analog ribavirin form the basis of the treatment of this infection but are not considered sufficiently effective and cause several side effects. In this study, we developed a novel viral-specific drug delivery method. Enveloped viruses, including HCV, expose an anionic phospholipid, phosphatidylserine (PS), on their surface to mediate their binding and entry into cells for infection. To target such exposed PS on HCV, we developed a chimeric recombinant protein containing human IFN and mouse lactadherin (also known as milk fat globule epidermal growth factor \(\beta\)), which binds with high affinity to PS. The IFN–lactadherin fusion protein showed a high binding affinity toward PS and HCV and consequently blocked viral replication in the infected cells more efficiently than conventional IFN. Overall, these data suggest that conjugation with lactadherin facilitates the delivery of any protein drug to PS-exposing enveloped viruses.

Key words drug delivery; enveloped virus; phosphatidylserine; lactadherin; interferon; viral replication

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-sense, single-stranded RNA virus belonging to the hepacivirus genus of the Flaviviridae family.\textsuperscript{1} Globally, chronic infection with HCV is considered a major healthcare problem, affecting 3% of the world population, \textit{i.e.}, approximately 170–200 million individuals.\textsuperscript{2}\textsuperscript{2} Since the identification of HCV, antiviral therapy based on interferon (IFN)-\(\alpha\), administered alone or in conjunction with the nucleoside analog ribavirin, has formed the basis of treatment regimens.\textsuperscript{3} IFNs are a multigene family of inductive cytokines that stimulate cells \textit{via} membrane receptors.\textsuperscript{4} Typically, type I IFNs are produced early upon virus infection as a first-line defense.\textsuperscript{5} These IFNs induce hundreds of IFN-stimulated genes, thereby inhibiting various stages of viral replication and enhancing host response\textsuperscript{6}; in addition, type I IFNs have some immunoregulatory activities.\textsuperscript{7} However, these treatments are frequently associated with various adverse effects, including influenza-like symptoms, hematological changes, and neuropsychiatric disturbances.\textsuperscript{8} Therefore, the development of a novel method in which IFN exhibits a more specific action on viruses is expected.

Several recent findings suggested that enveloped viruses expose negatively charged phospholipids such as phosphatidylserine (PS), which play a role in mediating virus entry.\textsuperscript{9–11} The presentation of PS on the outer leaflet of enveloped viruses disguises them as apoptotic bodies, thereby conning cells into engulfing the virions through cell clearance mechanisms. This mechanism of enhanced viral entry is termed as \textit{“apoptotic mimicry.”} PS is generally localized to the inner leaflet of plasma membranes but is redistributed and exposed on the outer membrane when a cell undergoes apoptosis.\textsuperscript{12} The exposure of PS on apoptotic cells provides a key signal that triggers cell engulfment. Apoptotic mimicry was first hypothesized to be used by hepatitis B virus.\textsuperscript{13} Later, the requirement of viral PS for efficient entry and infection was proposed for viruses such as human immunodeficiency virus (HIV), Ebola, Marburg, vaccinia, and Pichinde viruses.\textsuperscript{14} Recent findings indicate that even nonenveloped viruses have evolved strategies to engage apoptotic clearance receptors for internalization.\textsuperscript{15} It has been proposed that nonenveloped viruses such as hepatitis A virus and poliovirus become enveloped in the host cell membrane when they bud into the host cell organelles, \textit{i.e.}, multivesicular bodies or PS vesicles.\textsuperscript{16,18}

The soluble protein Gas6 binds to PS on the virion surface and bridges viruses to the cell surface by interacting with the tyrosine kinase receptor Axl; the formation of this complex is necessary for enhancement of virus entry.\textsuperscript{19} Various additional PS receptors have been identified to date, including T-cell immunoglobulin and mucin domain TIM-1 and TIM-4 proteins and lactadherin/integrin \(\alpha v/\beta 3\) or \(\alpha v/\beta 5\) complexes.\textsuperscript{17,20} Reportedly, the mutation of the residues involved in PS binding or complex formation of these receptors results in the inhibition of viral infection.\textsuperscript{21} Moreover, viral internalization into HEK293T cells was significantly enhanced by the overexpression of these PS receptors and was inhibited by competing with PS liposomes,\textsuperscript{22} providing evidence that virion-associated PS receptor interactions are responsible for virion uptake, and not just for virus binding. In particular, recent studies demonstrated that TIM-1 promotes HCV infection by serving as an attachment receptor for binding to PS exposed on the HCV envelope.\textsuperscript{23} The knockout of the TIM-1 gene or use of PS-containing liposomes resulted in a remarkable reduction of HCV cell attachment and subsequent infection. The property that HCV strongly exposes PS on its surface prompted the possibility that antiviral drugs such as IFN can be specifically delivered to the virus by targeting PS. Therefore, we employed a unique soluble protein (lactadherin) that strongly binds to PS.\textsuperscript{24} In this study, we developed a chimeric protein in which human IFN was conjugated to mouse
lactadherin that has a strong affinity toward PS and showed that the engineered protein can be a novel drug that efficiently prevents the replication of HCV by specifically targeting the IFN to the virus.

MATERIALS AND METHODS

Cloning of a Chimeric Gene of a Human IFN and Mouse Lactadherin cDNA A chimeric gene was constructed using recombinant PCR to express a fusion protein comprising N-terminal human IFN-β and C-terminal mouse lactadherin. Synthesized human IFN-β cDNA (FASMAC, Japan) was amplified by PCR using the following primer pair: IFN-forward (5′-ACC ATG ACC AAC AAG TGT CTC CT-3′) and IFN-reverse (5′-GGAGTACA GAA GTG CTTCC GAG GTT ACC-3′; the lactadherin sequence is underlined). Mouse lactadherin cDNA coupled to the FLAG-tag coding sequence was obtained by PCR using the pcAG–lactadherin (D89E) plasmid as a template in which the FLAG-tag sequence was conjugated to the 3′-end and an amino acid substitution was introduced in the RGD motif to inactivate phagocytosis, as described previously,20 with the following primer pair: lactadherin-forward (5′-GGTACCTCCGAAGACGACTTCGTGACCTC-3′; the IFN sequence is underlined) and lactadherin-reverse (5′-TTA CTT GTC GTC GTC GTC TGC TCTTGT-3′). Both PCR products were purified, mixed, and further amplified by the second PCR with the IFN-forward and lactadherin-reverse primers. Then, the chimeric IFN–lactadherin cDNA was inserted into the pcAG vector.

Purification of Lactadherin or the IFN–Lactadherin Fusion Protein pcAG–lactadherin (D89E) or pcAG–IFN–lactadherin fusion protein was transfected in HEK293T cells using polyethylenimine (PEI); PEI/DNA complexes were formed using a 10:1 PEI:DNA mixture by diluting 20 µg DNA in a 2 mL reduced serum medium (Opti-MEM, Gibco, U.S.A.)

Results

Development of the Recombinant IFN–Lactadherin Fusion Protein Lactadherin, or milk fat globule epidermal growth factor (EGF) 8 (MFG-E8), is a glycosylated protein that was first discovered as a component of milk fat globule membranes.23 Mouse lactadherin contains two EGF domains: proline/threonine-rich domain (P/T-rich domain) and two fac-

RESULTS

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Lactadherin binds to PS exposed on apoptotic cells, extracellular vesicles, and enveloped viruses via C-1 and C-2. Using this property, we developed a chimeric fusion protein in which mouse lactadherin was connected to human IFN-β. We used mouse lactadherin because the P/T-rich domain, which is absent in human lactadherin, contributes to affinity enhancement toward PS by 10 times. Because the RGD motif in the second EGF domain promotes phagocytosis by binding to the integrin αvβ3 or αvβ5 on phagocytes, we introduced an amino acid substitution (from D to E) in this motif. The protein is tagged with FLAG sequence in the C-terminus for affinity purification.

IFN–Lactadherin Has a High Affinity toward PS First, to examine the binding ability of IFN–lactadherin to PS, microtiter plates were coated with lactadherin (diamonds) or IFN–lactadherin (squares), and the protein bound to the wells was quantified by ELISA. Assays were performed in triplicates, and the average and standard deviation values are plotted. The proteins did not bind to the plates without PS-coating.

IFN–Lactadherin Efficiently Inhibits HCV Replication Based on the observation that IFN–lactadherin efficiently bound to HCV, we tested the effect of inhibiting virus replication. Serially diluted IFN–lactadherin or rhIFN-β protein was incubated with HCV. The virus–protein mixture was then added to hepatoma Huh-7.5.1 cells to check the effect of both proteins on viral replication. The results showed that incubation with IFN–lactadherin or rhIFN-β at a concentration of 400 nM substantially inhibited the replication of HCV at the same efficacy as quantified by QPCR (Fig. 4). However, when unbound proteins were washed away from the virus–protein mixture, the inhibition of viral replication by rhIFN-β was markedly impaired, whereas that by IFN–lactadherin remained unchanged. Moreover, even a lower concentration of IFN–lactadherin (100 and 25 nM) with a washing away step still retained the inhibitory ability, comparable to higher concentration (400 nM) due to the binding to HCV.

DISCUSSION In summary, our data indicated that IFN–lactadherin bound to HCV with a high affinity, which is not characteristic to conventional IFN. We initially thought that lactadherin would inhibit the uptake of viruses by masking PS on viral membrane, but even a high concentration of lactadherin did not inhibit the HCV infection (data not shown). Therefore, we employed the PS binding property of lactadherin to develop this drug delivery method to enveloped viruses. In this study, we washed viruses to remove unbound proteins from the virus–protein mixture. This process is intended to mimic the blood flow in the human body, assuming that blood flow washes IFN away from healthy organs (which are absent in viruses) and delivers IFN only to the site harboring viruses. The used proteins were detected using anti-IFN-β antibody by Western blotting (Fig. 3). We found that only IFN–lactadherin bound to the virus due to the ability of lactadherin to bind to PS exposed on the viral envelope.
lactadherin-conjugation method could be applied for specific delivery of any protein drug to enveloped viruses by targeting the PS exposed on their surface, which may provide a novel strategy for inhibiting infection by many viruses that use apoptotic mimicry and contribute to reduce the side effects caused by the drug.

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Conflict of Interest The authors declare no conflict of interest.

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