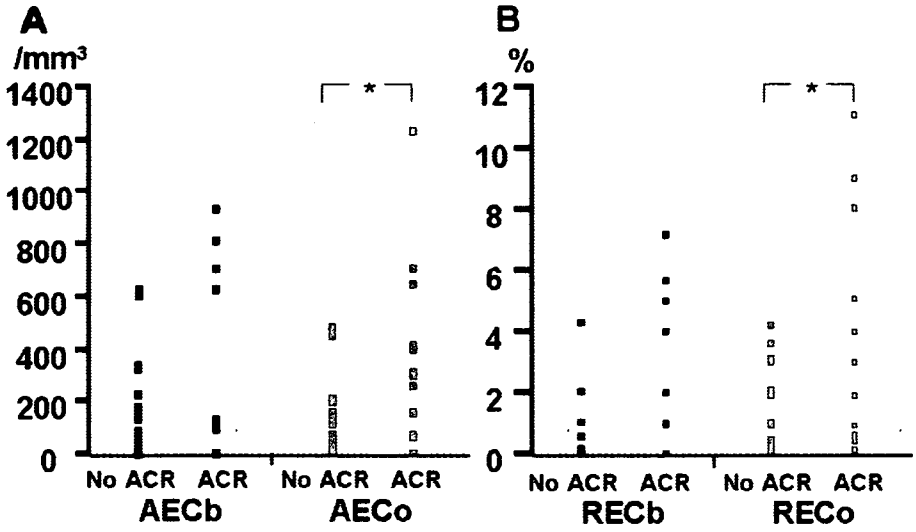

Fig 1. Distribution of absolute eosinophil counts (AEC, **A**) and relative (REC, **B**) in blood on 3 days before (black squares) and on the day of biopsy (grey squares). *$P < .0001$.

duct complication could be ruled out by ultrasonography, a biopsy was performed. ACR was diagnosed by experienced pathologists and graded into 4 classes according to the Banff scheme.[14]

Relative and absolute blood eosinophil counts obtained 3 days before the biopsy (RECb and AECb) and on the day of biopsy (RECo and AECo) were retrospectively reviewed. Transaminase levels on the day of biopsy were also reviewed. Eosinophilia was defined as REC ≥4% and/or AEC ≥400/mm³, as described previously.[6] Blood eosinophil data was successfully obtained from 91 (62%) biopsy samples from 45 patients.

Eosinophil counts of patients diagnosed with ACR were compared with those of patients without ACR. We then used a receiver operating characteristic curve to evaluate the sensitivity and specificity of eosinophilia in predicting ACR. All data were expressed as mean ± standard error. Statistical comparison of quantitative and qualitative data was performed using Wilcoxon's test and Fisher exact test, respectively. $P < .05$ was considered to be statistically significant.

RESULTS

Of 91 biopsies, ACR was indeterminate in 71 cases (non-ACR group) and mild and moderate ACR was confirmed in 18 and 2 cases, respectively (ACR group). Aspartate aminotransferase levels on the day of biopsy were not significantly different between the ACR and non-ACR groups (97 ± 16 IU/L vs 111 ± 9 IU/L, respectively; $P = .35$). Similarly, there was no significant difference in alanine aminotransferase levels between the ACR and non-ACR groups (169 ± 26 IU/L vs 164 ± 14 IU/L; $P = .90$).

Figure 1 shows the eosinophil counts of the ACR and non-ACR groups. Although RECb and AECb levels were higher in the ACR group, the difference was not statistically significant ($P = .14$ or $P = .18$, respectively). In contrast, RECo and AECo levels were both significantly higher in the ACR group ($P < .0001$ for both comparisons). The receiver

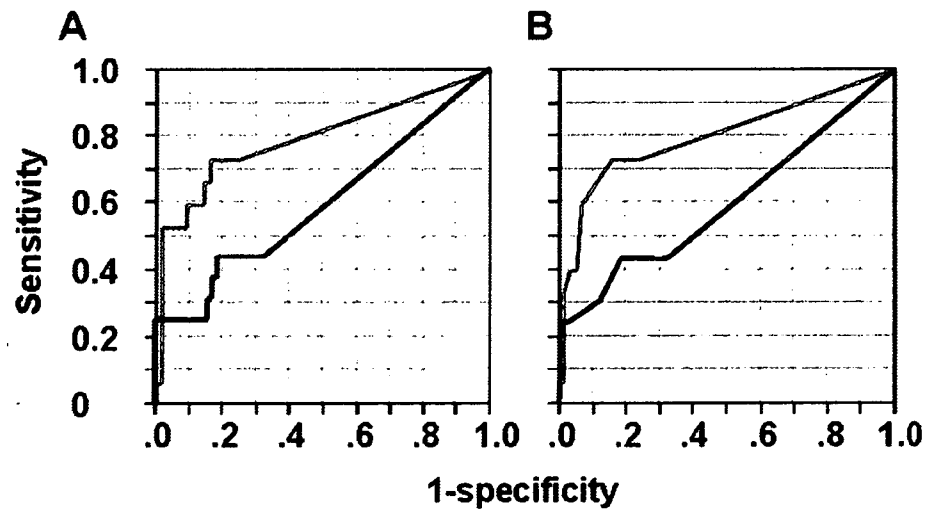

Fig 2. Receiver operating characteristic curve to evaluate the sensitivity and specificity of absolute **(A)** and relative **(B)** blood eosinophilia. Black line, eosinophil count 3 days before biopsy; grey line, eosinophil count on the day of biopsy.

Table 1. The Sensitivity and Specificity of Blood Eosinophilia to Predict Acute Rejection

	Sensitivity (%)	Specificity (%)
RECb ≥4%	20	100
AECb ≥400 cells/mm^3	20	100
RECo ≥4%	25	99
AECo ≥400 cells/mm^3	20	99
RECb ≥2%	25	89
AECb ≥200 cells/mm^3	20	96
RECo ≥2%	45	94
AECo ≥200 cells/mm^3	40	96

operating characteristic curve of the association between eosinophil counts and ACR is shown in Fig 2. The sensitivity and specificity of eosinophilia for predicting ACR is shown in Table 1. When the threshold of eosinophilia was set as REC ≥2% or AEC ≥200/mm^3, the sensitivity of eosinophilia for predicting ACR was 40% (REC) or 45% (AEC), whereas the specificity was 94% (REC) or 96% (AEC).

DISCUSSION

In the present study, we found that HCV-positive patients diagnosed with ACR had significantly higher REC and AEC levels on the day of biopsy than HCV-positive patients without ACR. These results indicate that measures of blood eosinophil levels might contribute to the differential diagnosis of ACR in HCV-positive recipients.

Few studies have evaluated whether postoperative eosinophilia predicts ACR.[15] Nagral et al[16] reviewed 129 biopsy cases after deceased donor liver transplantation. They reported that AEC levels 1 or 2 days before, or on the day of biopsy, predicted ACR with low sensitivity (30%–38%) and high specificity (83%–92%). Our current results are consistent with these findings; however, the present study was specifically limited to HCV-positive patients. The occurrence of eosinophilia was equal in comparison with 140 ACR episodes of HCV-negative patients in our subjects (33% vs 34% for relative blood eosinophilia, 27% vs 29% for absolute blood eosinophilia). The suggestion by Barnes et al[12] that the eosinophil response is suppressed in the presence of HCV was not supported by our study.

Recurrent HCV and ACR cannot be completely differentiated because the histological findings are similar or even overlapping between the 2 pathologies.[17] It remains controversial whether serum HCV-RNA level indicates the severity of hepatitis[18] of allografts. For differentiation, C4d, an end-product of the activated classical complement cascade, is a useful marker of ACR[19]; a high HCV-RNA titer in liver tissue[20] or increased anti-HCV immunoglobulin (Ig)M[21] are markers of HCV recurrence. Among these, both C4d and anti-HCV IgM had high specificity for predicting ACR and HCV recurrence, respectively (91% and 100%), but the sensitivity was low (68% and 82%), similar to that of the eosinophil counts in the present analysis.

In conclusion, evaluation of blood eosinophil count can be helpful for confirming ACR. REC ≥2% or AEC ≥200 cell/mm^3 convincingly suggest ACR with a specificity of 94% or 96%, respectively.

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Small RNA Molecules as Therapeutic Agents for Viral Infectious Diseases

[1]Jing Zhang and [2]Toshio Hattori
[1]Research and Development Center,
FUSO Pharmaceutical Industries, Ltd., Osaka, Japan
[2]Department of Infectious and Respiratory Diseases,
Tohoku University School of Medicine, Sendai, Japan

Abstract: The potential of using small RNA molecules as therapeutic agents has been extensively explored, antisense RNA, ribozyme, aptamer, decoy and more recently siRNA have been demonstrated to be highly efficient in inhibiting a number of pathogenic viruses including human immunodeficiency virus, hepatitis B and C virus, poliovirus and influenza virus. The specificity and potency of the sequence-specific agents such as antisense, ribozyme and siRNA in particular, imply that these strategies will prove to be promising therapeutics for treating viral infections, although the antiviral efficacy may be limited by emergence of escape variants. Distinct from the reagents targeting viral RNA, decoy and aptamer inhibit viral replication by binding and thus inactivating the viral component such as regulatory gene product and viral enzyme. This review provides an up-to-date overview of the progress and problems in small RNA-based antiviral approaches, with a focus on their therapeutic utility, delivery and unwanted side effects.

Key words: Antisense RNA, antiviral approaches, ribozyme, aptamer, decoy, siRNA, therapeutics utility

INTRODUCTION

Despite much effort towards preventing viral infectious disease, chronic infection with viruses such as Human Immunodeficiency Virus (HIV), hepatitis B and C viruses (HBV and HCV) have been increasing, remaining serious worldwide health problems. Additionally, the emerging of avian influenza virus that can infect humans and the outbreak of the Severe Acute Respiratory Syndrome (SARS) caused by SARS coronavirus imply the threat of a global virus pandemic. The approaches to combat viral infections include vaccine and drugs that are targeted to specific viral enzymes or other proteins. One unavoidable problem is selection of resistant mutants during long-term treatment and multiple targets are generally required to prevent the emergence of mutant variants.

The concept of using RNA molecules as therapeutic agents for viral infection has aroused increasing interest in the recent decade. Antisense strategies, which encompass antisense oligonucleotides, ribozymes and small interfering RNA (siRNA), involve small nucleic-acid-based molecules that inhibit viral replication in a sequence-specific manner. The antisense reagents can be designed to target any viral RNA provided its sequence is known, making them theoretically ideal antiviral therapeutics. However, because of the strict sequence-specific property intrinsic in these approaches, the possibility of emergence of escape mutants upon persistent treatment is concerned. Other RNA-based strategies for combating viral disease include apatmer and decoy, which inhibit viral replication by binding and consequently inactivating the viral component such as regulatory gene product and viral enzyme.

Corresponding Author: Jing Zhang, Research and Development Center, FUSO Pharmaceutical Industries, Ltd., Osaka, Japan Tel: 81-6-6969-3131 Fax: 81-6-6964-2706

Antisense RNAs

Antisense oligonucleotides are single-strand DNA or RNA oligomer of 18-25 nucleotides in length, designed to bind to its target RNA via Watson-Crick base pairing. Depending on the type of antisense oligonucleotides used, there are two different action modes involved in gene knockdown. Conventional oligonucleotides such as phosphodiesters and phosphorothioates, recruit cellular RNase H to the duplex to cleave the target RNA; later-generated oligonucleotides including the modified oligonucleotides derivatives such as morpholinos, locked nucleic acids and peptide nucleic acids, do not active RNase H but inhibit translation by steric hindrance instead. In addition to their application in analysis of gene function, intensive studies have been conducted to explore the potential of antisense oligonucleotides as therapeutic modalities for diseases caused by the expression of deleterious genes, especially in the field of cancer and viral infections.

Viruses with the RNA genomes are particularly well suited to be targeted by antisense oligonucleotides, because both the mRNA and genomic RNA can be targeted, destruction of viral RNA could eliminate not only viral protein synthesis but also viral replication. On the other hand, in the case of DNA viruses or retroviruses, only mRNA can be targeted by antisense nucleotide, making virus clearance theoretically impossible and persistent treatment necessary. The first attempt employing antisense nucleotides as the viral therapeutic was published in 1978, which reported an inhibitory effect of oligodeoxynucleotides on replication of Rous-Sarcoma virus (Zamecnik and Stephenson, 1978). Since then, a lot of studies have demonstrated that antisense oligonucleotides are effective in fighting many pathogenic viruses. Theoretically, antisense oligonucleotides can be designed to target any region of viral RNAs, however, in view of the genetic diversity among viral isolates, it is necessary to target viral RNA sequences that are conserved and normally invariant among different strains. Actually, the 5'-untransalted region (UTR) is one of the most highly conserved regions in the HCV genome and has most frequently been targeted with antisense oligonucleotide. The 5'-UTR region, however, constitutes the internal ribosome entry site (IRES) capable of initiating cap-independent translation of the viral protein. Highly ordered RNA structures and multiple sites participating RNA-protein interaction within 5'-UTR have been documented to be crucial for translation and/or replication, both of which make the RNA inaccessible by oligonucleotides, extensive efforts were made to identify effective target sites.

In addition to mediating degradation of the target RNA, antisense oligonucleotides have been designed to inhibit essential processes of viral life cycle by steric blockade. For example, chimeric 2-*O*-methyl/LNA oligoribonucleotides against trans-activation response element (TAR) of HIV, which interacts with Tat trans-activator protein and cellular factors to stimulate transcriptional elongation, were reported to block HIV transcription by steric hindrance of the tat-TAR interaction (Arzumanov *et al*., 2001). Additionally, it was reported that antisense RNA targeting the splice donor-packaging signal of HIV inhibited viral replication via inhibition of both viral protein synthesis and virion RNA packaging (Chadwick and Lever, 2000).

Antisense oligonucleotides are usually delivered with cationic lipid reagents that are positively charged and capable of neutralizing the negative charge of the oligonuleotides. One of the major challenges of antisense oligonucleotide approach is the stabilization of oligomers, since single-stranded nucleic acid molecules are unstable and are degraded in blood stream with a few hours. A number of chemically modified nucleotides have been employed to enhance nuclease resistance. Phosphorothioates are the major representatives of first generation of modified oligonucleotide. Phosphorothioates are advantageous over unmodified oligonucleotides in resistant to nucleolytic degradation, but they also have undesirable feature such as decreased binding affinity to target RNA and propensity to bind to various protein, which may result in toxic side effects (Levin, 1999). Second generation oligonucleotides represented by 2'-*O*-methyl and 2'-*O*-methyoxy-ethyl RNA were developed to solve these problems, they are less toxic and have enhanced affinity with target RNA,

but the unsatisfied feature is that they cannot activate RNase H to cleave the target RNA. More recently, novel chemically modified nucleotides, so-called third generation oligonucleotides, have been developed. Most of them exhibit improved properties such as enhanced stability, higher target affinity and lower toxicity. Further, gapmers with a stretch of unmodified or phosphorothioate DNA monomers in the center of the oligonucleotide are widely used to overcome the shortcoming of inability to activate RNase H.

Although nearly 20 antisense oligonucleotides have progressed to the stage of clinical trials, only one drug was approved by the Food and Drug Administration, which is used to treat cytomegalovirus-induced eye infection in AIDS patients. Most clinical trials were interrupted because of the unsatisfactory effectiveness.

Ribozymes

Ribozymes, catalytic RNAs that are capable of cleaving target RNA, are another important category of sequence-specific gene-silencing molecules. Since the discovery of the first group I nitron ribozymes in the early 1980s, a variety of ribozymes, including hammerhead and hairpin ribozymes, have been developed. Ribozymes have a catalytic domain that is flanked by sequences complementary to the target RNA. The hammerhead ribozyme was first isolated from viroid RNAs, it can be transformed from a *cis*-cleaving molecule into a target-specific *trans*-cleaving enzyme by dissecting the catalytic and substrate strands of the ribozyme. Hammerhead and hairpin ribozymes are the most intensively studied ribozymes. Similar to those for antisense nucleotides, the problems that should be cleared in developing therapeutic ribozymes are: 1) accessible target sites have to be selected; 2) the oligoribonucleotides have to be stabilized against nucleolytic degradation and 3) the ribozymes have to be delivered to target cells.

Hammerhead ribozyme consists of two substrate binding arm and a catalytic core cleaving any NUH triplets (where N can be any ribonucleotide and H can be any ribonucleotide except guanosine) with AUC and GUC triplets being processed most efficiently. Hairpin ribozymes usually cleave after BNGUC (where B can be any nucleotide except adenosine). Because of secondary and tertiary structures of the target RNAs, not all sequences that are theoretically cleavable by ribozymes can be served as the target sites for efficient cleavage. Computer predictions of the secondary structure of the target RNA and systemic experiments with a number of antisense oligonucleotides or ribozymes have been made to identify accessible target sites. Another approach to facilitate the access and subsequent cleavage of the ribozyme was reported by Taira and coworkers, who developed novel ribozymes with the ability to access any target site regardless of the secondary structure by combining with the unwinding activity of the endogenous RNA helicase eIF4AI (Kawasaki and Taira, 2002). In addition to the secondary structure, cellular compartmentalization of target RNAs is also thought to influence their susceptibility to ribozyme cleavage. Enhanced cleavage efficacy was reported by co-localization of the viral RNA and ribozyme tethered with the retroviral packaging signal (Sullenger and Cech, 1993).

As the delivery of ribozymes, chemically synthesized ribozymes can be exogenously introduced into the target cells using the reagent as described for antisense oligonucleotides. However, stabilization of ribozymes is more difficult than that of antisense oligonucleotides, since introduction of modified nucleotides most likely causes conformational changes that attenuate its catalytic activity. Indeed, a number of attempts exploiting uniform structural modifications to enhance nuclease resistance of ribozymes were demonstrated to be infeasible due to the reduced catalytic activity (Paolella *et al*., 1992; Pieken *et al*., 1991; Shimayama *et al*.,1993). A systemic study of a variety of modified hammerhead ribozymes led to the identification of a consensus ribozyme motif with enhanced nuclease resistance while maintaining the catalytic activity by keeping the 5 purine ribonucleotides in the catalytic core unmodified (Beigelman *et al*., 1995). In addition to directly introducing the synthesized one, ribozyme can also be endogenously expressed from plasmids inside the target cells, which can

elicit constant and long-lived ribozyme expression. Because RNA polymerase III (pol III) promoter is highly productive and capable of generating complex RNA structures with high integrity, pol III promoters such as the tRNA promoters are widely used to direct the expression of both hammerhead and hairpin ribozymes (Medina and Joshi, 1999; Yamada *et al*., 1994).

Ribozymes have widely been used to inhibit virus replication. Combined with retrovirus system, hammerhead and hairpin ribozymes direct target various HIV-1 regions were demonstrated to be effective in inhibiting viral replication (Zhou *et al*., 1994; Ojwang *et al*., 1992). Another ribozyme-based anti-HIV approach was accomplished by cleavage of the chemokine receptor CCR5 or CXCR-4 and thus perturbing their coreceptor function (Goila and Banerjea, 1998). Further, the protective effect of ribozymes against HIV-1 infection has also been demonstrated *in vivo*. Using the SCID-hu mouse *in vivo* human thymopoiesis model, $CD34^+$ hematopoietic progenitor cells transduced by retrovirus expressing anti-tat-rev and -env ribozymes and Rev aptamers were showed significantly resistant to HIV-1 infection upon challenge (Bai *et al*., 2002).

In addition to HIV-1, the potential to use ribozymes as tools to control HCV infection has also been studied. Extensive knowledge of IRES structure and high conservation among HCV genotypes have rendered the IRES element attractive as the target for ribozymes. Chemically synthesized hammerhead ribozymes targeting the conserved sites of HCV IRES significantly inhibited (>90%) the replication of HCV/poliovirus chimera (Macejak *et al*., 2000). Adenoviral vectors have been considered to be an attractive candidate to deliver anti-HCV ribozymes because of the hepatotropic property, it was reported that adenovirus-delivered anti-HCV ribozymes were effective at eliminating HCV RNA in infected primary human hepatocytes (Lieber *et al*., 1996). Simultaneous expression of multiple ribozymes targeting different conserved HCV RNA region from a single vector was attempted to circumvent the emergence of resistant viral mutants (Welch *et al*., 1998).

Encouraged by successful inhibition of viral replication in cell culture and *in vivo*, several ribozymes have subsequently been tested in clinical trials. The first clinical trials was conducted with retroviral-delivered hammerhead and hairpin ribozymes against HIV-1 RNA and another one used chemically synthesized hammerhead ribozyme targeting HCV (Hepatazyme), but unfortunately, both of which had to be quitted because of the poor therapeutic efficacy (Michienzi *et al*., 2003; Peracchi, 2004).

RNA Interference

In the past few years, research in the antisense field was revolutionized by discovery of RNA interference (RNAi). RNAi is an evolutionarily conserved phenomenon of posttranscriptional gene silencing that has been described in plants, invertebrates and vertebrates. When double-stranded RNAs (dsRNAs) are introduced into these organisms, they are cleaved into small interfering RNAs (siRNAs) of 21-23 nt by the endonuclease Dicer, followed by incorporation of siRNA into a RNA-Induced Silencing Complex (RISC), which unwinds the duplex and uses the antisense strand as a guide to seek and degrade homologous RNA. In mammals, however, introduction of long dsRNA (>30 bp) induces systemic, nonspecific inhibition of translation due to activation of the interferon response. A breakthrough was achieved by the finding that specific gene silencing in mammalian cells can be mediated by siRNAs of 21 nt, which can bypass dsRNA-induced nonspecific interferon response (Elbashir *et al*., 2001). This finding triggered numerous studies using siRNA in mammalian cells. RNAi is the most potent antisense reagent discovered thus far, it was reported that siRNA-mediated gene silencing is about 100-1000 fold more efficient than that by antisense oligonucleotides (Bertrand *et al*., 2002).

Similar to ribozymes, siRNA can be either introduced as synthetic short dsRNA molecules or intracellularly transcribed from plasmids. siRNA is of relatively high stability and efficient siRNA delivery and silencing can be achieved by use of the cationic lipid reagent, but the silencing effect

mediated by exogenously introduced siRNA is short-lived. When longer lasting gene silencing is desired, plasmids or viral vectors are employed to deliver siRNA expression cassette. The pol III promoters of small nuclear RNA U6 and the H1 RNA component of RNase P have been widely used to direct the siRNA expression. Double-stranded RNA molecules can be expressed separately as sense and antisense RNA using two promoters or transcribed as short hairpin RNAs (shRNAs) which are then processed to give siRNAs. Inducible knockdown of gene expression was achieved by incorporated the doxycycline-responsive element into pol III promoter (Van de Wetering *et al*., 2003) or by coupled with Cre-loxP recombination system (Kasim *et al*., 2004). Additionally, it was reported that transport of shRNAs from the nucleus to the cytoplasm is likely to be an event involving Exportin-5, a karyopherin participating in the nuclear export of pre-microRNA, thus efficient nuclear export could be obtained by artificial modification to render the loop sequences of shRNA analogous to that of pre-microRNA (Yi *et al*., 2003).

The first study using siRNA as a antiviral reagent was reported by Bitko and Barik (2001), who demonstrated an inhibitory effect of synthetic siRNAs direct target viral polymerase and fusion protein F on respiratory syncytial virus. Afterwards, many studies have described RNAi-mediated inhibition of a large variety of viruses. One common approach in siRNA-based antivial strategy is to directly target key RNA sequences within viral genome. Inhibition of HIV-1 has been achieved by siRNAs directed against tat and rev (Coburn and Cullen, 2002; Lee *et al*., 2002), reverse transcriptase (Surabhi and Gaynor, 2002), trans-acting response region (TAR), 3'-UTR and vif (Jacque *et al*., 2002). Similarly, replication of HCV replicon RNA was suppressed by siRNAs targeting the capsid and nonstructural protein (NS) 4B (Randall *et al*., 2003), NS3 and NS5B (Wilson *et al*., 2003; Kapadia *et al*., 2003), 5'-UTR (Yokota *et al*., 2003). Besides these two viruses, siRNA-based antiviral strategy has also been successfully applied to other pathogenic viruses, including poliovirus, dengue virus, influenza virus, SARS coronavirus and many others. In addition to direct targeting viral sequence, another approach is to target host proteins considered to be crucial for the life cycle of viruses. It was reported that siRNA targeted to HIV-1 receptor CD4 (Novina *et al*., 2002), or co-receptor CCR5 (Qin *et al*., 2003) block the entry and replication of HIV-1. Also, evidence obtained from our group and those from others showed that knock down of cellular co-factors polypyrimidine tract binding protein, La antigen and human VAMP-associated protein of 33 kDa inhibits HCV RNA replication (Zhang *et al*., 2004; Domitrovich *et al*., 2005; Gao *et al*., 2004). Cellular genes are less prone to mutation and antiviral approach by knock down host factors is therefore less likely to allow viral escape of silencing. However, the unintended side effects of knocking down cellular gene are concerned and must be addressed thoroughly prior to therapeutic application.

By demonstrating that a great variety of viruses can be successfully targeted by siRNAs, it is conceivable that these powerful antisense molecules can be used to target any preexisted or newly emerging human pathogenic virus. However, siRNA-mediated antiviral technology faces several important challenges that must be circumvented. Like other sequence-specific antisense reagents, one outstanding drawback of the approach using siRNA direct against viral genome is that emergence of siRNA-resistant variants. In fact, it was shown that a single point mutation in the siRNA target region conferred escape in poliovirus (Gitlin *et al*., 2002) and more recently HIV-1 was shown to elude siRNA targeting by the evolution of an alternative structure in RNA genome (Westerhout *et al*., 2005). Additionally, increasing evidence showed that viruses counteract RNAi effect by encoding viral proteins that act as the suppressor of RNAi pathway (Bennasser *et al*., 2005; Li *et al*., 2002). Several strategies may be useful to prevent the escape of mutant variants, for example, 1) simultaneous expression of multiple anti-viral siRNAs targeting different conserved viral sequences, 2) combination with siRNA against cellular co-factors indispensable for viral replication. Indeed, Schubert *et al*. (2005) reported that the silencing effect of the vector doubly expressing two different siRNAs was maintained even after artificially introducing a point mutation that disabled the respective mono-expression vector.

Although siRNAs are considered to be of high specificity for their targets, unwanted off-target effects may occur by siRNA recognition of other mRNAs with partially complementary sequence. Moreover, siRNAs can act like microRNAs to inhibit translation if there is a consecutive base pairing between siRNA and mRNA. Therefore, to minimize potential off-target effects, it is important to carefully comparing the candidate siRNA sequences with mRNAs in the human genome to avoid long stretches of homology.

Another potential toxicity of siRNA is that endogenous RNAi pathway, which is important in regulating the expression of developmentally essential genes, may be competitively inhibited by exogenously introduced siRNA, because the RNAi machinery such as Dicer and RISC may be limit in amount (Bitko *et al*., 2005). This is especially in the cases that high siRNA dose is administrated or multiple siRNAs are simultaneously expressed to avoid escape viral mutants.

As mentioned above, outstanding progress has been made since RNAi was shown to work in mammalian cells three years ago. The first phase I clinical trials targeting the VEGF angiogenic pathway in age-related macular degeneration have begun. Although the issues such as delivery and unwanted side effects are still the problems to be further cleared to turn RNAi from an effective functional genomics tool into a potent antiviral therapy, the prospects for overcoming these are good as we improve our understanding of the RNAi mechanism.

RNA Aptamers and RNA Decoys

RNA aptamers are short RNA ligands with binding and inhibitory activity to small molecule or protein targets. The screening and identification of such molecules with unique binding properties from very large random RNA libraries are generally accomplished by a technology termed systemic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold, 1990). The SELEX process starts with a large library of randomized RNA sequences containing 10^{14}-10^{15} different RNA species. The library is incubated with the target protein of interest and the RNAs that bind the protein are separated, amplified, cloned and sequenced. The high affinity and specificity of aptamers make them attractive therapeutic agents and increasing evidence indicated that the use of SELEX technique could generate aptamers to many disease relevant targets. The therapeutic utility of aptamers has been studied in a variety of human maladies including cancer, cardiovascular disease and infectious diseases.

In the case of using aptamers for antiviral purpose, the proteins essential for viral replication such as regulatory protein and viral enzyme are good target. Indeed, it was reported that RNA aptamers selected by SELEX against HIV-1 Tat (Yamamoto *et al*., 2000) or reverse transcriptase (Joshi and Prasad, 2002) inhibited HIV-1 replication by up to 99%. Similarly, RNA aptamers specific for HCV NS3 (Hwang *et al*., 2004; Nishikawa *et al*., 2003) or RNA-dependent RNA polymerase (Biroccio *et al*., 2002; Vo *et al*., 2003) were shown to block the replication of HCV replicon by inhibiting the viral enzymatic activities. In addition to vial proteins, antiviral aptamers were also selected to target key viral RNA sequences. Nishikawa and coworkers reported an inhibitory effect on IRES-directed translation by RNA aptamers binding to HCV IRES sequences (Kikuchi *et al*., 2005).

Another approach using small RNAs to target pathogenic protein is RNA decoys. RNA decoys are small RNA molecules analogous to *cis*-acting element, which can compete with the corresponding endogenous sequences for *trans*-factors binding, thereby attenuating the authentic *cis-trans* interaction. Since the concept of using decoys as therapeutic agents emerged 15 years ago, increasing evidence has shown that the decoy approach may be useful in the treatment of a variety of human disease. The first study performed to determine if an RNA decoy could be used to inhibit the activity of a pathogen protein was published by Sullenger *et al*. (1990) who demonstrated cells over expressing TAR decoy are highly resistant to viral replication. Later, same group reported that over expression of RRE-derived decoys inhibited HIV-1 replication by preventing the binding of Rev protein to the vial RNA (Lee *et al*., 1992). Also, it was demonstrated that RNA decoys mimicking the Stem-Loop (SL)

structures of HCV 5'-UTR inhibited IRES-dependent translation (Ray and Das, 2004). Additionally, considering that the SL structures in the NS5B coding region were demonstrated to function as *cis*-replicating elements (CREs) and replication of HCV is likely initiated by interaction between replicase complex and SL structures containing CREs, we explored the possibility of using RNA species corresponding such SL structures as antiviral decoys and provided the evidence showing that pol III-directed over expression of SL RNA inhibited HCV replication by sequestering the replication complex and preventing its binding to the physiological target in the viral RNA (Zhang *et al*., 2005).

Distinct from sequence-specific antisense reagents, the efficacy of antiviral approach by aptamers and decoys may be less affected by the extensive variability encountered among viral isolates. Aptamer resistance may be less of a problem because RNA-protein interactions are not easily disrupted by mutations in the protein. Indeed, the effective cross-clade inhibition of HIV-1 by gp120 aptamers (Khati *et al*., 2003) and to a lesser extent by reverse transcriptase (Joshi and Prasad, 2002) was documented. Additionally, because the interaction between *cis*-acting element and *trans*-acting factor is usually essential for viral replication and mutation in the *trans*-acting factor that blocks its binding to RNA decoy also blocks its binding to the authentic target in viral RNA. To circumvent RNA decoy-mediated inhibition, double mutation, one in *trans*-acting protein and another compensatory one in the *cis* element is simultaneously required, thus making the chance of emergence of escape mutants lower.

To provide the resistance to nuclease degradation in biological fluids, aptamers are routinely selected with amino-or fluoro-modifications at the 2' position of pyrimidines which are prone to nuclease attack. Further stabilization of selected RNA aptamers includes *O*-methyl-substitutions in purine nucleotides, which requires chemically synthesis of modified RNA molecules. An alternative to chemical modification is the application of L-nucleic acids during and after *in vitro* selection. Modified aptamers can be delivered with the same reagents used for antisense oligonucleotides, the half-life of aptamers in the plasma is increased by coupling with high molecular linkers such as PEG. Similar to ribozymes and siRNAs, both aptamers and decoys can also be intracellularly expressed from plasmid-or vector-delivered expression cassette. Intracellularly expressed aptamers (intramers) and decoys could elicit long-term and stable effect, which is particularly essential for antiviral purpose.

Despite of the high binding specificity and affinity, the efficiency of aptamer-or decoy-mediated inhibition may be not efficient enough in some cases. This can be improved by combination with other antisense modalities. Because aptamers and decoys aim at inhibition of protein function, whereas ribozyme and siRNA destroy target protein-encoding mRNA or viral RNA; so the additive or synergistic antiviral effect can be expected by combining these two conceptually different reagents.

Another issue that limits the therapeutic use of aptamers is relatively high cost for aptamer manufacturing and delivery of aptamers and decoys faces the same problems as with other antisense reagents. Further progress in aptamer and decoy therapeutic field will depend on the breakthrough of such problems.

CONCLUSION

Small RNA-based antiviral approach represents a useful alternative to small molecular compounds for combating viral infection. Antisense oligonucleotides and ribozymes have been used for many years to inhibit viral replication. The therapeutic efficiency of antisense oligonucleotides and ribozymes was far from satisfied. The more recently developed RNAi strategy, however, is obviously advantageous over the older generation antisense reagents due to the high efficiency in knockdown gene expression. RNAi field is moving at an unprecedented speed and the first clinical trials using RNAi have already commenced. The problem for emergence of siRNA resistant variants can be dissolved either by simultaneously targeting different conserved region or combination with other small RNA-based modalities such as aptamer and decoy, which bind and subsequently inactivate target protein. With the

advances in developing efficient delivery systems, one could expect that RNAi and other small RNA-based approaches can become realized as effective therapies to treat viral infections in the near future.

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Clathrin-Dependent Entry of Severe Acute Respiratory Syndrome Coronavirus into Target Cells Expressing ACE2 with the Cytoplasmic Tail Deleted▽

Yuuki Inoue,[1] Nobuyuki Tanaka,[1,4]* Yoshinori Tanaka,[1] Shingo Inoue,[3] Kouichi Morita,[3] Min Zhuang,[2] Toshio Hattori,[2] and Kazuo Sugamura[1]

Department of Microbiology and Immunology[1] and Department of Infectious and Respiratory Diseases,[2] Internal Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan; Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan[3]; and Division of Immunology, Miyagi Cancer Center Research Institute, Natori 981-1293, Japan[4]

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The penetration of various viruses into host cells is accomplished by hijacking the host endocytosis machinery. In the case of severe acute respiratory syndrome coronavirus (SARS-CoV) infection, viral entry is reported to require a low pH in intracytoplasmic vesicles; however, little is known about how SARS-CoV invades such compartments. Here we demonstrate that SARS-CoV mainly utilizes the clathrin-mediated endocytosis pathway for its entry to target cells by using infectious SARS-CoV, as well as a SARS-CoV pseudovirus packaged in the SARS-CoV envelope. The SARS-CoV entered caveolin-1-negative HepG2 cells, and the entry was significantly inhibited by treatment with chlorpromazine, an inhibitor for clathrin-dependent endocytosis, and by small interfering RNA-mediated gene silencing for the clathrin heavy chain. Furthermore, the SARS-CoV entered COS7 cells transfected with the mutant of ACE2 with the cytoplasmic tail deleted, SARS-CoV receptor, as well as the wild-type ACE2, and their entries were significantly inhibited by treatment with chlorpromazine. In addition, ACE2 translocated into EEA1-positive early endosomes immediately after the virus attachment to ACE2. These results suggest that when SARS-CoV binds ACE2 it is internalized and penetrates early endosomes in a clathrin-dependent manner and that the cytoplasmic tail of ACE2 is not required for the penetration of SARS-CoV.

Severe acute respiratory syndrome (SARS) is an emerging infectious disease with high mortality caused by infection of the respiratory system by SARS coronavirus (SARS-CoV). SARS-CoV is a human enveloped coronavirus containing a helical nucleocapsid structure composed of a single-stranded, positive-polarity RNA of approximately 29 kb plus the poly(A) tail (44). Phylogenetic analysis classifies the coronaviruses into three groups based on their genetic and serological relationships.

The first essential step in virus infection is the entry of viruses into host cells. After their attachment to their respective cell surface receptor, most viruses make use of cellular endocytosis machineries, such as clathrin-dependent and -independent pathways, for their entry (22, 29). Clathrin-dependent endocytosis has been well characterized using growth factor receptors such as the transferrin receptor (23), epidermal growth factor receptor (EGFR) (39), and the keratinocyte growth factor receptor (3). The endocytosed receptors are translocated into endosomes, where they are degraded or recycled to the cell surface. Similarly, various viruses, among them Semliki Forest virus, vesicular stomatitis virus (VSV), and influenza virus, enter into host cells via the clathrin-dependent endocytosis pathway and translocate into endosomes, where they are uncoated (10, 35, 40). The clathrin-dependent endocytosis is initiated by the binding of adaptor protein 2 (AP2) complexes to the cytoplasmic tail of the cell-surface receptors, which recruits clathrins (27, 38). Subsequently, the receptors are invaginated to form "pits," which are surrounded by a spherical cage-like structure made of clathrin triskelions. Viruses bound to the receptors are endocytosed similarly and then transported to vesicles called early endosomes. It is well known that early endosomes are somewhat acidic (pH 6.5 to 6.0) and become more acidic as they mature to form late endosomes (pH 6.0 to 5.5). The acidification of endosomes is required for incorporated viruses to establish an infection (22, 29).

On the other hand, the clathrin-independent pathways include a caveola-dependent pathway. Caveolae are relatively smaller vesicles of 50 to 100 nm in diameter, formed by membrane invagination at the cell surface, and coated by caveolin-1 (12, 15, 28). Simian virus 40 (SV40), for example, utilizes caveolae to be internalized into the "caveosomes" under a neutral condition (30). In contrast, effective internalization of SV40 was also found in cells that do not express caveolin-1, suggesting that SV40 utilizes not only the caveola-dependent pathway but also the lipid-raft-dependent and caveola-independent pathway (6). Other viruses utilizing the caveola-dependent pathway include some of the picornaviruses (21), papillomaviruses (4), filoviruses (8), and retroviruses (2).

The binding and subsequent entry of SARS-CoV into the host cells are primarily mediated by a viral spike glycoprotein, called S protein, which binds to its receptor, angiotensin-converting enzyme 2 (ACE2) (16). ACE2 is a cell-surface-bound enzyme of the type I membrane protein topology, with its

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Sendai, 980-8575 Japan. Phone: 81-22-717-8096. Fax: 81-22-717-8097. E-mail: n-tanaka@mail.tains.tohoku.ac.jp.
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catalytic site exposed to the extracellular surface. ACE2 along with its related family protein ACE is indispensable for blood pressure homeostasis via the renin-angiotensin system (31). However, despite the 40% amino acid identity shared by ACE and ACE2, ACE does not act as a SARS-CoV receptor (25). The broad expression profile of ACE2, which includes the gastrointestinal tract and lungs, matches well with the affected organs in patients with SARS (9). Although accumulating evidence has documented a physical and functional interaction between SARS-CoV and ACE2, little is known about how the ACE2-mediated entry of SARS-CoV is linked to cellular endocytosis machineries.

In the present study, we investigated the internalization mechanisms of SARS-CoV after binding to ACE2. We found that SARS-CoV hijacks the clathrin-dependent machinery for endocytosis via ACE2 with the cytoplasmic tail deleted, as well as the wild-type ACE2.

MATERIALS AND METHODS

Cell lines. The cell lines used here were human hepatoma HepG2 and monkey kidney COS7 cell lines, which were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS) and antibiotics, under 5% CO_2 in a humidified incubator.

Virus and infection. SARS-CoV (Vietnam/NB-04/2003) was maintained in Vero E6 cell cultures as described previously (43). In brief, the SARS-CoV titer of the stock virus was determined by infection of Vero E6 cells by a 50% tissue culture infective dose. Cells were inoculated by SARS-CoV at a multiplicity of infection of 1 and allowed to adsorb the virus for 1 h at 4°C. Subsequently, the cells were washed with phosphate-buffered saline (PBS) and cultured for the indicated times. All experiments using infectious SARS-CoV was done in a laboratory certified with biosafety level 3.

RNA extraction and RT-PCR. Total RNA from HepG2 cells infected with SARS-CoV was extracted with the TRIzol reagent (Invitrogen Corp.) by the following the protocol. Reverse transcription-PCR (RT-PCR) for the detection of viral RNA was performed with Titan One-Tube RT-PCR kit (Roche Molecular Systems) by following the manufacturer's protocol. The primer sequences for SARS-CoV detection were gained from World Health Organization network laboratories. The sequence of BNIoutS2 (sense) is 5′-ATGAATTACCAAGTCAATGGTTAC-3′, and the sequence of BNoutAs (antisense) is 5′-CATAACCAGTCGGTACAGCT AC-3′. For an internal control, a primer set of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used. The sequence of GAPDH-F (sense) is 5′-AGTC AGCCGCATCTTCTTTTGC-3′, and the sequence of GAPDH-R (antisense) is 5′-CTCCTGGAAGATGGTGATGGGA-3′.

Pseudoviruses. The pseudoviruses were SARS-CoV(HIV), VSV(HIV), and A-MLV (amphotropic murine leukemia virus)(HIV), which consist of the viruses' respective envelope glycoprotein, the HIV Gag/Pol proteins, and a luciferase plasmid. The pseudoviruses were produced with methods described previously (41). In brief, 293T cells were transfected with 7 μg of pCMVΔR8.1 (HIV Gag/Pol plasmid) and 7 μg of pHR′-luciferase, along with an expression vector for the respective viral envelope glycoprotein: 800 ng of pCMV-SARS-S (SARS-CoV S plasmid), 7 μg of pMD.G (VSV G plasmid), or 7 μg of pDJ (A-MLV envelope glycoprotein plasmid). The 293T cells were cultured in 10-cm dishes, and transfection was performed by the calcium phosphate method. At 48 h posttransfection, the culture supernatants were harvested and filtered through 0.45-μm-pore-size screens. These filtered supernatants were used to infect host cells with the pseudoviruses.

Preparation of ACE2 mutants. Three mutants of ACE2 were prepared from pcDNA ACE2 expression vector (20). ACE2-Δtail mutant has a stop codon at the end of the transmembrane domain, ACE2-Δtail-TM mutant is ACE2-Δtail mutant replaced with the transmembrane domain derived from EGFR, and ACE2-sol consists of the extracellular domain of ACE2. The extracellular domains of ACE2-sol from pcDNA ACE2 were amplified by PCR and introduced into pCXN2 after XhoI digestion. These plasmids were transfected with FuGeneHD according to the manufacturer's instructions (Roche Molecular Systems).

Luciferase assay. Cells were infected with pseudovirus for 12 h, and the culture medium was then replaced with virus-free Dulbecco modified Eagle medium. After further 48 h of incubation, the cells were lysed for luciferase assays. The Dual-Luciferase Reporter Assay System (Promega) was used according to the manufacturer's protocol. The luciferase activities of the samples were measured with a Lumat LB 9507 (Berthold).

Inhibition of endocytosis and endosomal acidification. Extraction of cholesterol from the plasma membranes was performed by using methyl-β-cyclodextrin (MBCD; Sigma), as described elsewhere (7). HepG2 cells seeded in a 24- or a 96-well plate 1 day prior to the experiments were treated with serially diluted MBCD for 30 min. After extensive washes with ice-cold PBS, pseudoviruses were added in the absence of MBCD for an additional 12 h. For the inhibition of clathrin-mediated endocytosis, cells were incubated with the respective doses of chlorpromazine (Sigma) for 1 h and then infected with the pseudovirus for an additional 12 h in the presence of chlorpromazine. After extensive washes with PBS, cells were further incubated in the absence of chlorpromazine for 48 h. To determine the pH dependency of the pseudoviruses, the cells were pretreated with serial dilutions of an endosome acidification inhibitor, either NH_4Cl (Sigma) for 1 h, and then infected with pseudoviruses in the presence of the inhibitor. Luciferase activities were determined for cell extracts prepared from these cultures 48 h after the infection.

Immunoblotting. Immunoblotting assays were performed as described previously (17). In brief, cells were lysed in NP-40 lysis buffer (1% Nonidet P-40, 40 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μg of aprotinin/ml). The cell lysates were precleared of cellular debris by centrifugation (10,000 × g) for 30 min at 4°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were electrotransferred onto polyvinylidene difluoride membranes (Millipore). The membranes were first blocked with 5%% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and then probed with the indicated primary antibodies. The antibodies used were anti-human CHC (Santa Cruz), anti-α-tubulin (Sigma), and anti-ACE2 (R&D Systems). After three washes, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Signals were visualized with a Super Signal Pico Detection kit (Pierce), and digital images were collected with a Lumi-Imager F1 (Roche Molecular Systems).

Depletion of CHC by siRNA. To transfect small interfering RNA (siRNA) designed to target the clathrin heavy-chain gene (Invitrogen Corp.), a reverse transfection method was used by using Lipofectamine RNAiMAX (Invitrogen Corp.) according to the manufacturer's instructions. In brief, 5 pmol of stealth siRNA duplex-Lipofectamine RNAiMAX complexes were used for 10^5 HepG2 cells in a 24-well plate. Gene silencing efficiency was assessed 48 h after the transfection by immunoblotting. The siRNA (clathrin heavy chain [CHC]) target sequence was 5′-UGAAGUAUUGACAUCAAAUUUCCGG-3′. A nonfunctional oligonucleotide was used for the control (Invitrogen Corp.).

Immunofluorescence staining. HepG2 cells were grown in 35-mm glass-bottom dishes (Iwaki) at a density of 4×10^4 cells/dish. After 24 h, the cells were washed twice with PBS, fixed with 4%% paraformaldehyde for 15 min, washed with the wash buffer (0.1% Triton X-100–PBS), and blocked with the same buffer containing 10% FCS. For immunostaining, samples were incubated overnight with the primary antibodies, including anti-ACE2 and anti-EEA1 (Santa Cruz). After three washes, the samples were incubated with the secondary antibodies (Molecular Probes; Alexa 488-labeled and Alexa 594-labeled antibodies) for 1 h. Confocal imaging was performed by using the 510 META microscope with a 60×/1.30-0.60 oil immersion objective lens (Carl Zeiss).

RESULTS

Effects of endocytosis inhibitors on SARS-CoV entry. To investigate the entry mechanism of SARS-CoV, we used SARS-CoV(HIV), because it allows safe, rapid, and quantitative analyses. Two other pseudoviruses, the VSV(HIV) and A-MLV(HIV), were used as control viruses, because VSV(HIV) utilizes the pH-dependent and clathrin-dependent entry mechanisms (40), while A-MLV(HIV) utilizes the pH-independent and clathrin-independent entry mechanisms (2, 25, 40).

HepG2 cells were infected with pseudoviruses, SARS-CoV-(HIV), VSV(HIV), or A-MLV(HIV) in the presence of an endocytosis inhibitor, chlorpromazine. Chlorpromazine is a cationic amphiphilic drug that disrupts clathrin-mediated endocytosis by inhibiting the relocation of clathrin and AP2 from the cell surface (42). HepG2 cells were pretreated with various

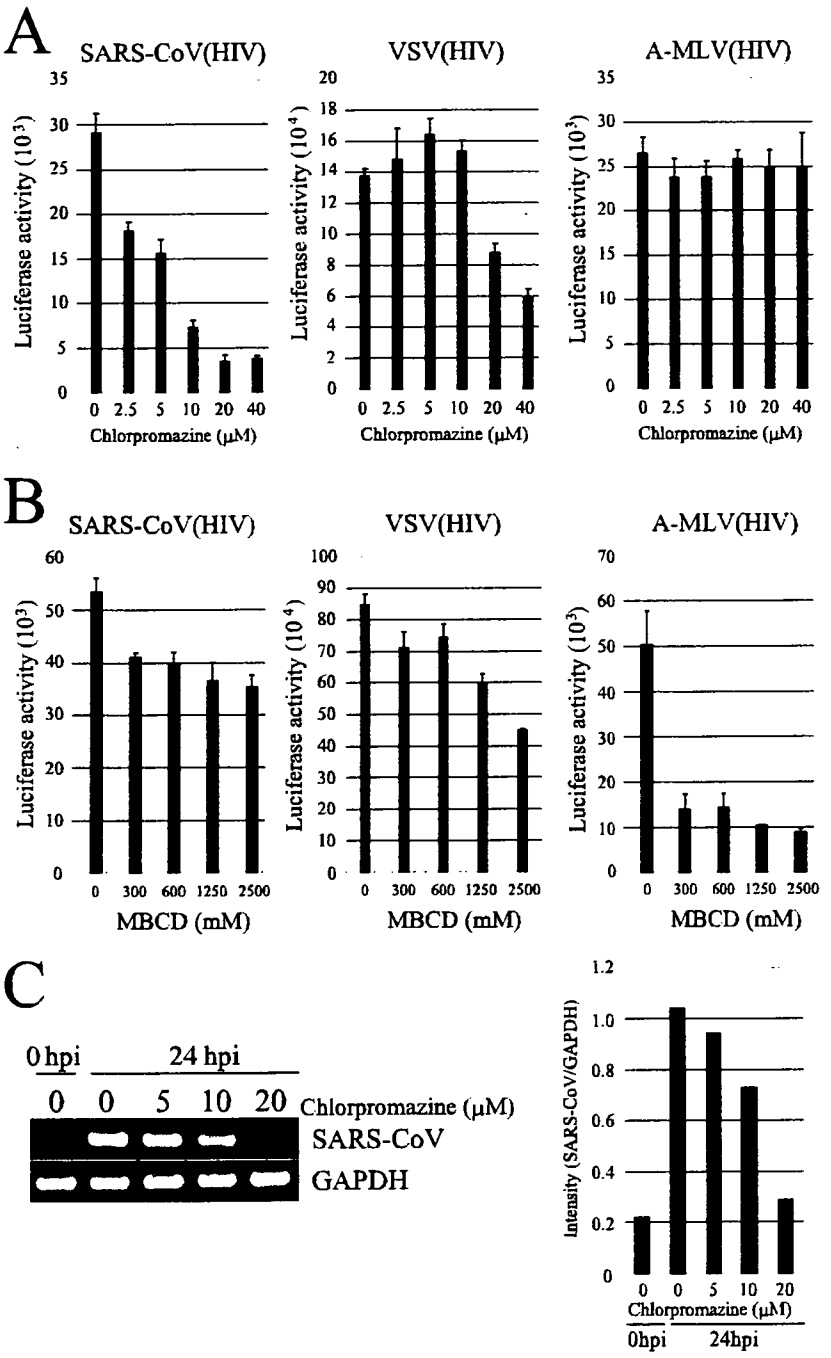

FIG. 1. Effects of chlorpromazine and MBCD on pseudoviruses and SARS-CoV infection. (A and B) HepG2 cells were treated with the indicated doses of chlorpromazine or MBCD and then infected with SARS-CoV(HIV), VSV(HIV), or A-MLV(HIV) for overnight. The effects of chlorpromazine and MBCD on the infectivity of each pseudovirus were evaluated by measuring the luciferase activities. The columns represent the mean values of triplicate experiments; bars indicate maximum values. (C) HepG2 cells treated with the indicated doses of chlorpromazine were infected with SARS-CoV (Vietnam/NB-04/2003) for 24 h, and their expressions of viral RNA were measured by RT-PCR.

doses of chlorpromazine for 1 h and then infected with the indicated pseudovirus in the presence of chlorpromazine (Fig. 1A). Chlorpromazine significantly inhibited the infection efficiency of SARS-CoV(HIV); only 14% infectivity was observed with 20 μM chlorpromazine. Infection by VSV(HIV) was also inhibited, but less so; 42% infectivity was still seen with 20 μM chlorpromazine. Unlike the other two pseudoviruses, infection by A-MLV(HIV) was largely unaffected by any concentration of chlorpromazine.

We also examined the effects of another endocytosis inhibitor, MBCD, on infections by the pseudoviruses, because MBCD disrupts cholesterol-rich microdomains, resulting in

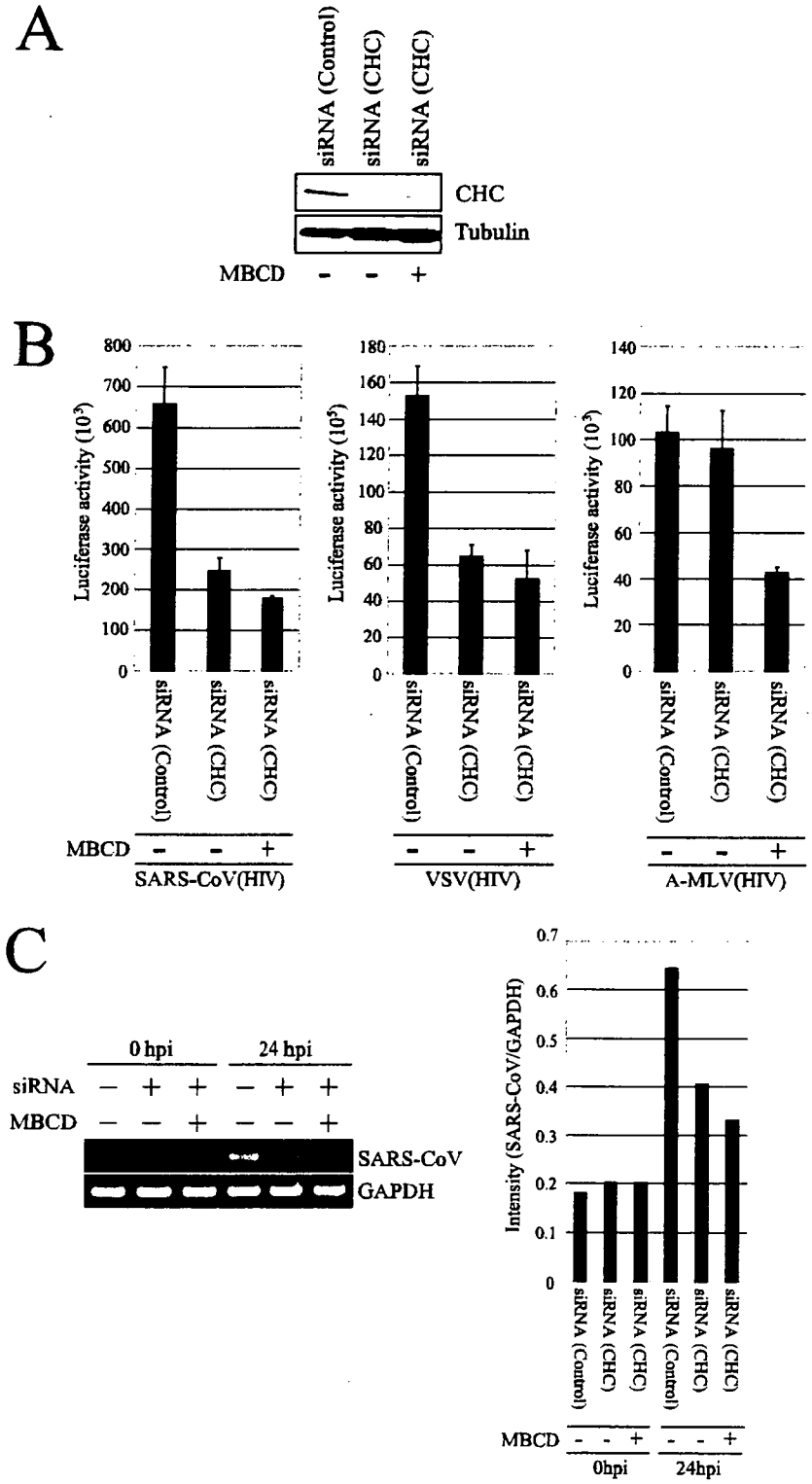

FIG. 2. Effects of CHC-specific siRNA on pseudoviruses and SARS-CoV infection. (A) HepG2 cells were transfected with the CHC-specific siRNA or the control siRNA and analyzed by immunoblotting with anti-CHC or anti-tubulin monoclonal antibody after 48 h of transfection. (B) The cells were treated with 2.5 mM MBCD for 30 min and then infected with each pseudovirus. The infectivities of the pseudoviruses are represented as luciferase activities. The experiment was performed in triplicate. (C) The cells were also infected with SARS-CoV (Vietnam/NB-04/2003) for 24 h, and their expressions of viral RNA were measured by RT-PCR.

the inhibition of both the caveola-dependent endocytosis and caveola-independent lipid-raft-dependent endocytosis (18, 32). HepG2 cells were treated with various concentrations of MBCD for 30 min and thereafter infected with the pseudoviruses in the absence of MBCD to avoid any potential effect of MBCD on the viral envelopes (Fig. 1B). MBCD treatment inhibited the susceptibility of HepG2 cells to A-MLV(HIV), to 18% of the control level at 2500 μM MBCD, whereas only a modest reduction in the cells' susceptibility to the SARS-CoV-(HIV) and VSV(HIV) was seen, to 66 and 53% of the control, respectively (Fig. 1B).

We next used SARS-CoV (Vietnam/NB-04/2003). HepG2 cells were treated or untreated with indicated concentrations of chlorpromazine for 1 h and then infected with SARS-CoV for 24 h. Infection efficiency of SARS-CoV was determined by RT-PCR. It was significantly inhibited by chlorpromazine treatment (Fig. 1C). Collectively, these results suggest that SARS-CoV entry into HepG2 cells is mostly mediated by the clathrin-dependent pathway, although some SARS-CoV pseudovirus entry appears to be dependent on caveolae and/or lipid rafts.

Effect of CHC depletion on SARS-CoV entry. To examine whether clathrin-mediated endocytosis is required for SARS-CoV(HIV) entry, we used siRNA-mediated gene silencing against the major component of the clathrin triskelion, CHC. In addition, under this clathrin knockdown condition, we simultaneously inhibited the clathrin-independent pathways with MBCD. This method was designed to determine whether the pseudoviruses entered the cells via the clathrin-dependent or -independent pathways, or both.

Transfection of a specific siRNA for CHC reduced the CHC protein expression to less than 10% of that in the control siRNA-transfected HepG2 cells, and we observed no effect of MBCD treatment on the CHC knockdown (Fig. 2A). CHC-depleted MBCD-treated cells, CHC-depleted mock-treated cells, and control cells were infected with the SARS-CoV(HIV), VSV(HIV), and A-MLV(HIV). CHC depletion reduced the SARS-CoV-(HIV) infectivity to 38% and the infectivity of VSV(HIV) to 42% of that in the control cells. Cholesterol depletion by MBCD in the CHC knockdown cells reduced the SARS-CoV(HIV) infectivity to 28% and the infectivity of VSV(HIV) to 36% of their infectivity in the control cells (Fig. 2B). On the other hand, there was little effect on the A-MLV(HIV) infectivity in cells expressing the siRNA. However, MBCD treatment markedly reduced the A-MLV(HIV) infectivity to 41% (Fig. 2B). The *t* test revealed the statistical significances in combinations between MBCD-treated and -untreated CHC knockdown cells infected with the pseudoviruses.

We also examined the effect of clathrin knockdown on SARS-CoV (Vietnam/NB-04/2003) infection to HepG2 cells under a similar condition to the pseudoviruses. CHC depletion reduced the SARS-CoV infectivity to 65% of that in the control cells at 24 h postinfection. Cholesterol depletion by MBCD in the CHC-knockdown cell slightly inhibited to this effect (Fig. 2C). These results support the evidence described above that the clathrin-mediated pathway of endocytosis is required for an efficient SARS-CoV entry into HepG2 cells.

Receptor activities of ACE2 mutants for SARS-CoV infection. To further investigate the clathrin-dependent endocytosis of SARS-CoV, we sought to determine whether the cytoplasmic domain of ACE2 is required for interaction with AP2/

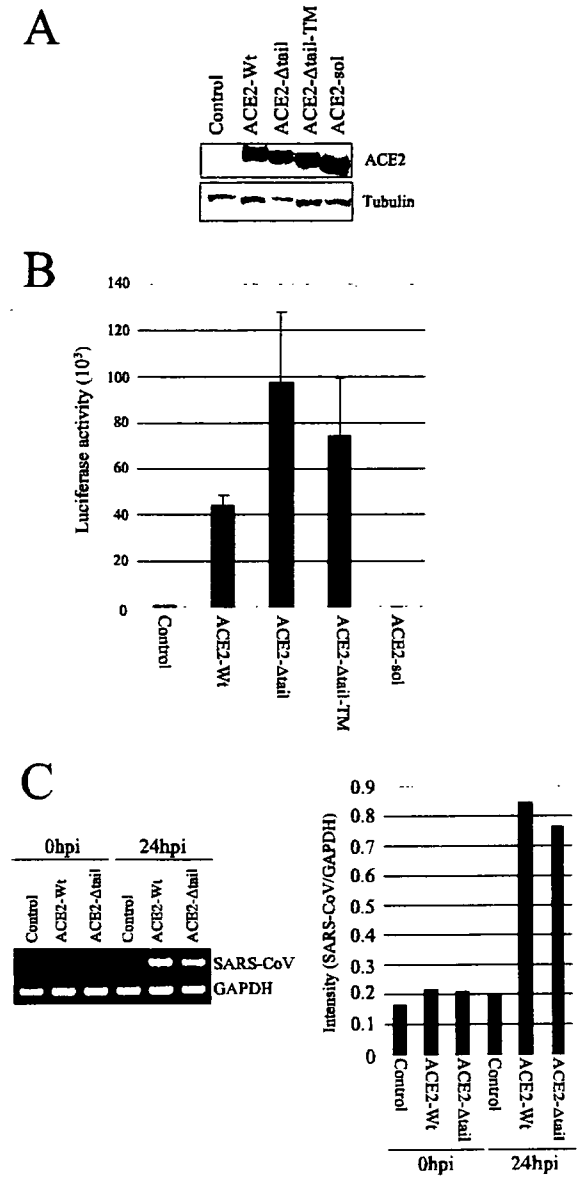

FIG. 3. Receptor activity of ACE2 mutants for pseudoviruses and SARS-CoV infection. (A) COS7 cells were transiently transfected with ACE2-wt, ACE2-Δtail, ACE2-Δtail-TM, ACE2-sol or control plasmids, and after 48 h of transfection their lysates were tested by immunoblotting with anti-ACE2 or anti-tubulin monoclonal antibody. (B and C) The transfected cells were infected with SARS-CoV(HIV) (B) or SARS-CoV (Vietnam/NB-04/2003) (C). Their luciferase activities were measured in triplicate, and their expressions of viral RNA were measured by RT-PCR.

clathrin complexes. We prepared an ACE2 mutant (ACE2-Δtail) that lacks the cytoplasmic domain by introduction of the stop codon at the end of the transmembrane domain of ACE2. The virus receptor activity of ACE2 mutant was examined with COS7 cells because COS7 cells are negative for ACE2 expression detected by RT-PCR and immunoblotting but positive for caveolin-1 (data not shown) (34). COS7 cells transfected with ACE2-Δtail were infected with SARS-CoV(HIV) equally well to the cells transfected with the wild-type ACE2 (Fig. 3A and

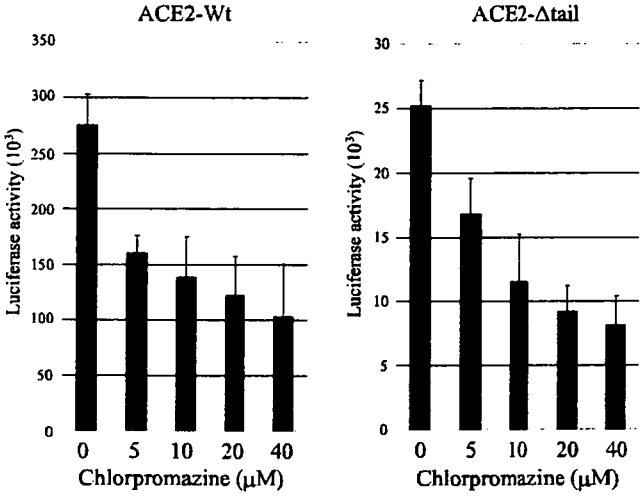

FIG. 4. Effects of chlorpromazine on pseudovirus infection to cells expressing ACE2-Δtail. COS7 cells were transiently transfected with ACE2-wt or ACE2-Δtail and then treated with the indicated amounts of chlorpromazine. Subsequently, the cells were infected with SARS-CoV(HIV). Their luciferase activities were measured in triplicate.

B). We further examined the receptor activity of ACE2-Δtail-TM, which lacks the cytoplasmic domain and replaces the transmembrane domain with that derived from EGFR. ACE2-Δtail-TM also showed a receptor activity for SARS-CoV(HIV) (Fig. 3B). We also confirmed that ACE2-sol, a soluble form of the ACE2 extracellular domain, has no receptor activity for SARS-CoV(HIV). Next, we used SARS-CoV (Vietnam/NB-04/2003). COS7 cells transfected with ACE2-Δtail were also infected with SARS-CoV equally well to the cells transfected with the wild-type ACE2 at 24 h postinfection (Fig. 3C). These results suggest that the cytoplasmic domain of ACE2 is not essential for its receptor activity and that there is no specificity of the transmembrane domain for its receptor activity.

We next confirmed that the ACE2-Δtail-mediated infection of SARS-CoV(HIV) is also clathrin dependent. COS7 cells transfected with the wild-type ACE2 or ACE2-Δtail were pretreated with chlorpromazine and infected with SARS-CoV-(HIV). The chlorpromazine treatment induced suppression of SARS-CoV(HIV) infection to COS7 cells expressing ACE2-Δtail, as well as the wild-type ACE2 (Fig. 4).

SARS-CoV(HIV) is transported into EEA1-positive early endosomes. Accumulating evidence suggests that cell surface molecules internalized by the clathrin-dependent pathway are transferred into early endosomes. We used confocal microscopy to examine whether ACE2 is internalized in early endosomes upon SARS-CoV(HIV) binding. After a 3 h period of serum starvation, HepG2 cells expressed ACE2 predominantly on the cell surface (Fig. 5, upper panels). The cells were then infected with SARS-CoV(HIV) concentrated 10-fold by ultracentrifuge. By 10 min after the infection, the ACE2 localization had changed dramatically, from the cell surface to EEA1-positive early endosomes (Fig. 5, lower panels). We also confirmed that the SARS-CoV(HIV) entry was affected by acidification inhibitors such as ammonium chloride (NH_4Cl) and chloroquine (data not shown). Furthermore, we examined the effect of ammonium chloride on SARS-CoV(HIV) infection into COS7 cells expressing ACE2-

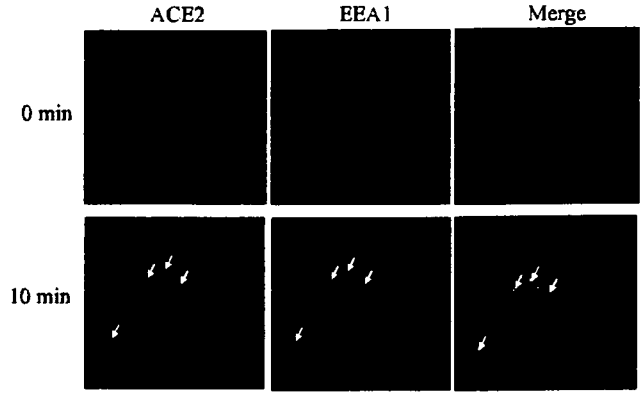

FIG. 5. Immunohistochemical localization of ACE2 after pseudovirus infection. HepG2 cells were cultured in the FCS-free medium to induce ACE2 on cell surfaces and incubated with concentrated SARS-CoV-(HIV) for 10 min at 37°C. They were then stained for ACE2 and EEA1 by immunofluorescence.

Δtail (Fig. 6). The ammonium chloride treatment induced inhibition of SARS-CoV(HIV) infection in a manner similar to that for HepG2 cells. These results suggest that the binding of the SARS-CoV(HIV) to ACE2 induces rapid internalization of the ACE2/pseudovirus complex into EEA1-positive early endosomes, where a low pH condition is required for it to establish an infection and that the cytoplasmic tail of ACE2 is not required for the internalization of SARS-CoV(HIV) into endosomes.

DISCUSSION

Productive infection of target cells by animal viruses requires their access to highly specific entry pathways that allow

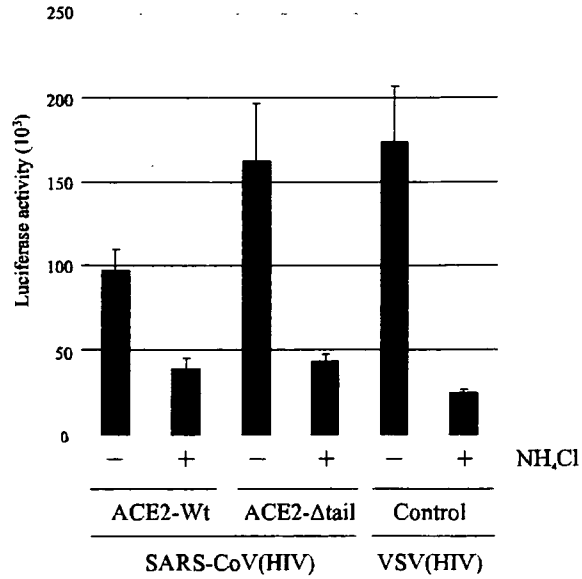

FIG. 6. Dependence on acidic environment for infection by the pseudoviruses. COS7 cells transiently transfected with ACE2-wt, ACE2-Δtail, or control plasmids were treated with 20 mM NH_4Cl and then infected with SARS-CoV(HIV) and VSV(HIV). Their infectivities were evaluated by measuring the luciferase activity. The experiment was performed in triplicate.