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Infectious diseases

Molecular targets for the rational design of drugs to inhibit SARS coronavirus

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Despite years of research, the precise determinants of coronavirus replication and pathogenesis remain unidentified. What is known of the pathogenesis of the severe acute respiratory syndrome coronavirus (SARS-CoV) is limited, but clinical observations suggest that both viral-induced cytotoxicity and host immune-mediated destruction contribute to the severity of disease. This summary discusses recent advances in coronavirus research that will facilitate the identification of crucial molecular targets for the rational design of SARS therapeutics.

Introduction

Historically, coronaviruses have not been regarded as severe human pathogens, as illness is primarily limited to mild upper respiratory infections that comprise 20% of the annual common colds worldwide [1]. However, the recent identification of a novel human coronavirus as the etiological agent of severe acute respiratory syndrome (SARS) dramatically demonstrated the potential of this virus family to cause devastating and potentially pandemic disease, as well as economic and social disruption [2]. Although coronavirus replication and pathogenesis has been the focus of many years of research by numerous

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When the SARS coronavirus came dramatically to attention as a fatal and potentially pandemic respiratory disease, the scientific response was swift and effective. The etiological agent was identified, the genome was cloned and sequenced, and large strides have been made in understanding key points in the viral life cycle, including recent identification of a functional host cell receptor and solution of the three-dimensional structure of the viral attachment protein. Much of this progress was possible only because a foundation of basic coronavirus biology existed. The Denison laboratory has been at the forefront of the small group of investigators who have dissected the basic features of coronaviruses. Denison has discovered critical mechanisms in the life cycle of the coronaviruses and is a key figure in the effective and urgent application of this knowledge to SARS.

dedicated investigators, many unanswered questions remain about exactly how these viruses proliferate in the host organism and bring about disease (see Outstanding issues). Of chief importance is the question: how can we inhibit coronavirus-induced diseases such as SARS? At present, there are no clinically proven treatments for SARS, and care of infected persons has been mostly empiric. This review gives a brief description of coronavirus replication and SARS coronavirus (SARS-CoV) pathogenesis, describes the current therapies for treating SARS, and highlights recent advances in the identification of crucial molecular targets for the rational design of antivirals and therapeutics.

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Main text

Disease manifestation and SARS-CoV pathogenesis

Patients diagnosed with SARS exhibit an atypical pneumonia with a lower respiratory tract infection, associated with pulmonary lesions, alveolar damage, mononuclear infiltrate and the presence of large multi-nucleated cells in the lungs. Although there have been no detailed studies of SARS-CoV pathogenesis, the clinical progression of SARS has been shown to follow a tri-phasic pattern [3]. Early-stage symptoms last approximately 7–10 days and include fever and general myalgia that is coupled with increasing viral load. At this phase, symptoms are thought to be primarily a consequence of viral-induced cytopathic effects. Mid-stage disease occurs between 10 and 14 days, revealing an increase in fever occurrence and diarrhea, and is accompanied by a slight decrease in viral load. Specific antibodies against SARS-CoV can be detected in patient sera at this stage of disease progression, suggesting that viral replication as well as host immune response might be contributing to illness. During late-stage infection a clinical worsening occurs that is associated with decreased viral shedding. Nearly 20% of patients suffer acute respiratory distress syndrome (ARDS) and require ventilatory support [3]. At late-stage infection, lung and immunopathological damage and severe lymphopenia have been proposed to be a direct result of an excessive immune response.

Because both viral replication and the host immune response are hypothesized to contribute to the pathogenesis of SARS-CoV, it is important to understand both aspects of virus biology to define potential therapeutics that combine direct antivirals and immune modulators. Viral factors influencing disease might include those that serve the sole purpose of enhancing virulence (much like toxins), as well as factors that cause cytotoxic effects on the host cell as a mere consequence of viral replication. To date, most of the individual SARS proteins have no known functions, and no potential virulence factors have been identified. Therefore, our discussion will be limited to the molecular determinants of coronavirus replication, the virus–cell interactions that occur during replication and their suspected contribution to pathogenesis, and the involvement of the immune system in enhancing the severity of coronavirus disease.

Contribution of viral-induced cytopathic effects to disease

It is not known how damage to infected cells caused by coronavirus replication impacts the severity of disease. The three main cytopathic effects of coronavirus replication on the host cell are: syncytia (multi-nucleated cell) formation, the initiation of apoptosis (programmed cell death), and the induction of cellular autophagy (degradation of intracellular components in lysosomes). For many coronaviruses, syncytia formation is a hallmark of infection during replication in cell culture. Syncytia form as a result of the cell–cell fusion caused

by viral attachment proteins (Spike or S), located on the infected cell surface, binding to receptors on adjacent cells. The lung pathology of SARS patients noted the presence of syncytia, however SARS-CoV does not cause Vero (African green monkey kidney) cells to become multi-nucleated in culture [3]. It remains unknown whether this cell–cell fusion event disrupts normal cellular processes, leading to cytotoxicity. Apoptosis is another potentially detrimental effect of coronavirus replication. One report showed an increase in the number of apoptotic cells in the spleen, lung and lymph nodes of SARS patients as compared with normal tissues [4]. The apoptotic cells include pneumocytes, lymphocytes and monocytes, which despite this increase in programmed cell death were still more numerous in infected tissues. Coronavirus-induced apoptosis has been studied in greater detail for the model coronavirus mouse hepatitis virus (MHV), specifically as it relates to the demyelination of the central nervous system (CNS) in mice. One study revealed that MHV induces apoptosis of oligodendrocytes upon entry of the virus into the cell and that this process does not require ongoing viral replication [5]. Presumably, the induction of programmed cell death is mediated either by signaling via a cell surface receptor that is activated upon virus attachment and entry, or is caused directly by a component of the incoming coronavirus virion. In support of the latter, it has been shown that expression of the SARS structural protein nucleocapsid in cultured cells leads to reorganization of the actin cytoskeleton and the induction of apoptosis [6]. It is not apparent whether the initiation of apoptosis is beneficial to coronavirus replication or instead just a by-product of viral entry. By contrast, activation of the host cell autophagy pathway, which is normally involved in protein and membrane recycling, is actually required for the efficient replication of MHV [7]. MHV, and probably all coronaviruses, induces the formation of intracellular double-membrane vesicles that serve as scaffolds for viral enzymes. These double-membrane vesicles are derived from host cell autophagosomes, and inhibiting components of the autophagy pathway disrupts viral replication. MHV infection increases the amount of cellular protein degradation by activation of autophagy. Consequently, this process could have severe cytotoxic effects on cells and contribute to viral pathogenesis.

Immunological factors enhancing coronavirus pathogenesis

The capacity of a virus to modulate the immune response can have a pivotal effect on the course of disease and the fate of the infected host. The severe lung inflammation, macrophage infiltration and lymphopenia seen in SARS patients suggests that the host immune response contributes significantly to the high morbidity and mortality of SARS. Still, it is not known why the immune system is so highly induced upon infection with SARS-CoV. It is probable that the adaptive immune response plays a role in SARS pathogenesis; however,

the robust activation of the innate response, specifically inflammation, seems to be the most devastating in terms of exacerbating disease. Elevated levels of proinflammatory cytokines, chemical signals that recruit immune cells to infected tissues, have been detected in SARS-CoV-infected persons. For example, pediatric SARS patients show markedly elevated levels of the cytokine IL-1 β , a primary mediator of inflammation that is also implicated in cellular apoptosis [8]. A recent study of adult SARS patients has demonstrated that as the severity of symptoms worsens, levels of the IL-6 (promotes antibody-producing B cells) increase, whereas levels of IL-8 and TGF- β (inhibits immune cell activation) decrease [9]. For MHV, innate immune responses are implicated in the neuropathology seen in infected mice. In particular, the capacity of different strains of MHV to differentially stimulate cytokine release in the CNS directs the type of infiltrating leukocyte populations and alters the severity of viral encephalitis [10]. The results of these studies support the contention that cytokine dysregulation during coronavirus infection might account, at least in part, for the severity of clinical disease.

Current therapies used for the treatment of SARS

To date, there are no clinically proven therapies for coronavirus infections, in animals or in humans. During the acute SARS epidemic of 2003, the severity and high mortality of the disease led to the use of numerous, and often combined empiric antiviral drugs and immune modulating therapies. Specifically, based on the clinical observations and inferences, patients infected with SARS-CoV were treated with steroids, to inhibit inflammation, and with the antiviral ribavirin, a nucleoside analog proven clinically effective for several other RNA viruses. The limitations of clinical and virological measures and the lack of controlled studies make it difficult to draw conclusions regarding the efficacy of ribavirin and steroid therapy to significantly lessen the severity of SARS. In a preliminary study, the use of interferon-alpha (INF α) in combination with corticosteroids was associated with a more rapid resolution of radiographic lung abnormalities [11]. Interferons inhibit viral infection both by activating immune cells and by altering the intracellular environment to restrict viral replication, thus making them good candidates for therapeutics. Moreover, one study reported that the administration of human immunoglobulin from convalescent patient sera showed some clinical benefits, suggesting that antibodies against SARS-CoV might decrease viral load [12]. Known antivirals targeting the viral enzymes, such as the proteinases, have also been tested as potential inhibitors of coronavirus replication. The cysteine proteinase inhibitor E64-d was shown to dramatically reduce SARS-CoV replication under cell culture conditions [13]. Additionally, the human immunodeficiency virus (HIV) aspartic acid protease inhibitor lopinavir-ritonavir decreased SARS-CoV

replication *in vitro* and was associated with reduced morbidity and mortality in infected patients when compared with untreated individuals [14]. Based on these preliminary reports, further evaluation of both E64-d and lopinavir-ritonavir using controlled studies is warranted.

Coronavirus replication and molecular targets for antivirals

Our current understanding of basic coronavirus biology, based on years of research by a cadre of committed investigators, has provided a molecular basis for the design of new antivirals. Specifically, the majority of what is known concerning intracellular coronavirus replication has been determined using MHV as a model system. Although the details of SARS-CoV replication remain to be determined, the similarities between MHV and SARS-CoV at level of the replicase proteins (enzymes that replicate the viral RNA) suggests that these two viruses can have similar intracellular replicative processes [15]. Thus, what we know about MHV can be applied to the development of drugs targeting the SARS-CoV replication machinery (Table 1).

Coronavirus replication is initiated by binding of the viral attachment protein (Spike or S) to specific receptors on the surface of the host cell and entry of the viral genome (positive-strand RNA) into the cytoplasm (Fig. 1). Molecules involved in viral attachment and entry represent the first targets for inhibition of the viral lifecycle. The type of receptor utilized by different coronaviruses is the primary determinant of the capacity of these viruses to replicate within a specific cell type or tissue. For SARS-CoV, angiotensin-converting enzyme 2 (ACE-2) has been identified as a functional receptor [16]. The fact that ACE-2 is expressed in numerous tissues in the human body supports the observation that SARS-CoV has been detected in the blood, lung, liver and kidneys. One report characterized the determinants of SARS-

Table 1. Targets for inhibiting SARS-CoV replication

Stage of virus lifecycle	Targets
Virus attachment and cell entry	Spike and ACE-2 ^a
Replicase protein processing	PLP ^b and 3CLpro ^c
Replication complex formation	ATG5 ^d and replicase proteins
RNA synthesis	RdRp ^e , Hel ^f and replicase proteins
Delivery of RNA to assembly sites	Hel ^f and nucleocapsid
Virion particle assembly	Viral structural proteins and cellular ER ^g proteins
Virion release from the cell	Viral structural proteins and exocytosis proteins

^a Angiotensin-converting enzyme-2, a cellular receptor for SARS-CoV [16].

^b Papain-like proteinase.

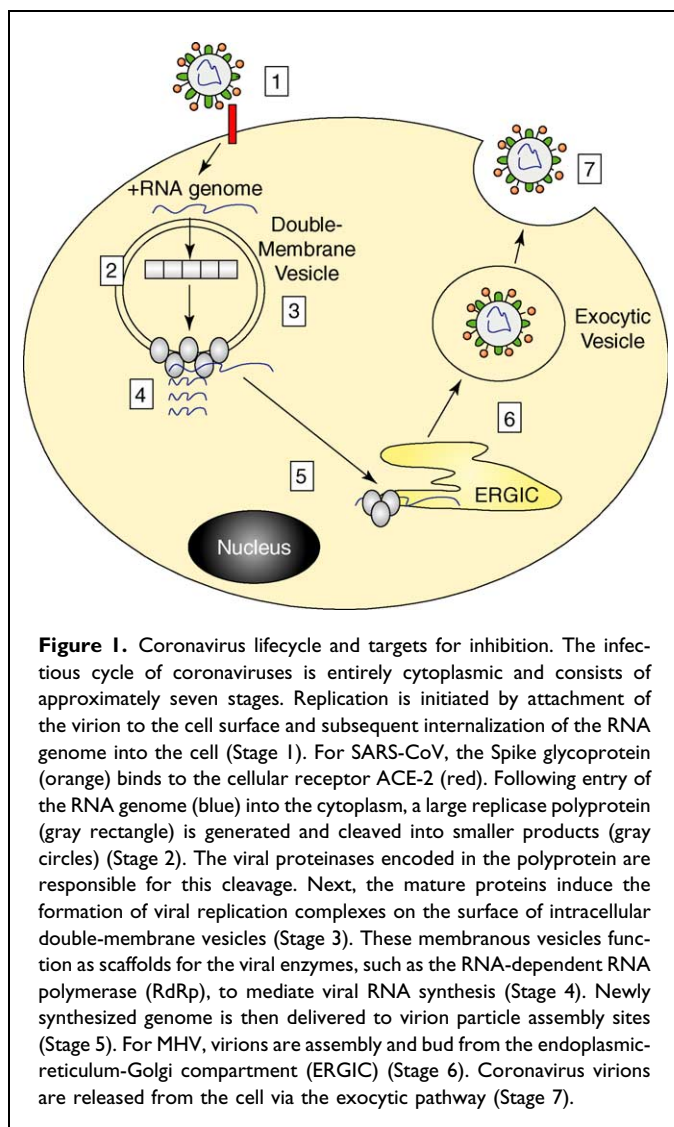
^c 3C-like proteinase.

^d Autophagy Gene 5.

^e Viral RNA-dependent RNA polymerase.

^f Viral helicase.

^g Endoplasmic reticulum.



CoV entry into cells and the three-dimensional crystal structure of Spike, the viral attachment protein, has been solved [17,18]. These studies provide the blueprints for the development of inhibitors of viral entry, probably targeting the Spike glycoprotein or the viral receptor ACE-2.

Following entry of the genome RNA molecules into the cytoplasm, the next stage in the virus lifecycle is generation of the viral proteins required for RNA synthesis. Enzymes or proteins involved in the synthesis of viral RNA can, in theory, be inhibited without detrimental effects on the host cell and are thus ideal targets for antivirals. Coronaviruses generate proteins required for viral RNA synthesis first as a large polyprotein, which is subsequently cleaved into smaller products by viral-encoded cysteine proteinases [1,15]. Among these mature protein products are an RNA-dependent RNA polymerase (RdRp), an RNA helicase, and several putative RNA processing enzymes [19]. It is hypothesized that mature proteins must be newly translated and processed to function in viral RNA synthesis. Specifically, the addition of proteinase

inhibitors at anytime during the MCV lifecycle abolishes viral RNA synthesis and halts replication [20]. These results, combined with the clinical and *in vitro* studies with proteinase inhibitors and SARS-CoV, suggest that the viral proteinases are principle targets for drug design. The determination of the crystal structure of the SARS-CoV main picornavirus 3C-like proteinase (3CLpro), facilitates the design of such drugs by elucidating determinants of inhibitor binding [21]. Moreover, a bioinformatics model of the catalytic core of the SARS-CoV RdRp has been proposed, allowing insight into the structure and function of this key viral enzyme during RNA synthesis [22]. Other important targets are proteins involved in the formation of membranous cytoplasmic viral replication complexes, which serve as scaffolds for the viral proteins to mediate RNA synthetic activities. Cells that have an engineered deletion of autophagy gene 5 (ATG 5) are unable to form both autophagosomes as well as viral-induced double-membrane vesicles. These cells do not support efficient viral replication, suggesting that components of the cellular autophagy pathway can be targets for antiviral development [7].

The assembly and release of coronavirus virions from the host cell is the final stage of the viral lifecycle. Although the molecular determinants of coronavirus assembly and release are not well characterized, these processes represent additional targets for inhibition of replication. Following viral RNA synthesis in the cytoplasm, newly synthesized genome RNA molecules are transported to sites of virion particle assembly by an unknown mechanism. For MCV, virus assembly and budding occur in the endoplasmic-reticulum-Golgi intermediate compartment (ERGIC) [23]. Several MCV proteins, including the helicase and the nucleocapsid protein, also translocate to the ERGIC during times of particle assembly, and are thought to be involved in the delivery of the RNA to be packaged into newly budding virus [24]. Thus, inhibiting the capacity of helicase and nucleocapsid to deliver genomic RNA molecules to the ERGIC would probably hinder particle assembly. Trafficking of assembled coronaviruses to the cell surface where they are released is not well understood, but is presumed to occur through the normal exocytic processes.

Summary and conclusions

Although the SARS outbreak is now under control, the likelihood of possible human and animal reservoirs suggest that this virus will continue to pose a worldwide public health threat. Understanding the biology and pathogenesis of coronaviruses is crucial to the development of antivirals and therapeutics for potential intervention in the case of a SARS-CoV reemergence. Such drugs will probably target factors involved in the cellular destruction caused by viral replication, as well as factors influencing immune-mediated damage. Highest priority should be given to the development

of antivirals that will block SARS-CoV replication. Inhibitors of early steps in the virus lifecycle could block early- and mid-stage cytopathic effect and possibly decrease immunopathological damage observed in SARS-CoV-infected persons.

Since the last identified naturally occurring case of SARS in July 2003, remarkable progress has been made in studies to identify targets for interference with virus replication, to identify potential therapeutics for SARS-CoV, to develop animal models for replication and pathogenesis, and to test potential vaccine candidates. In a recent comprehensive study more than 10,000 natural and synthetic compounds were assayed for the capacity to inhibit SARS-CoV replication in cell culture [25]. This screen identified approximately 50 putative antivirals that are currently in clinical development, suggesting that a cure for SARS might be closer than once thought. But the question that remains is – should the development and testing of therapeutics and vaccines be sustained, because SARS appears to have retreated into its unknown endemic host and is not causing human disease? The answer to that question is yes, for several reasons. First, there have been isolated cases of SARS since July 2003, resulting from lab-associated infection, but also possibly from *de novo* transmission from animal to human. As the recent cases from China attest, the very characteristics of SARS-CoV that resulted in the worldwide epidemic are the same ones that make it possible to reemerge, including a relatively long incubation period, nonspecific prodrome, and variably efficient transmission. Second, the natural host or mechanism for introduction and maintenance in humans is not known, and thus it is important to have strategies for implementation in the case of new outbreaks. Third, many of the strategies being tested have probably application to other coronaviruses of humans and animals, and thus are potentially important in their own right. Finally, there are discussions about SARS-CoV biosafety and biosecurity, both for inadvertent and possible intentional outbreaks. It is therefore essential to understand targets for therapeutics and to define strategies for prevention. In summary, the application of knowledge concerning the clinical manifestation of coronavirus disease and the molecular determinants of coronavirus replication will provide a foundation for the rapid development of effective inhibitors of these pathogenic viruses.

Outstanding issues

- How can we prevent or treat coronavirus diseases?
- How does damage to infected cells caused by coronavirus replication impact the severity of disease?
- What is the role of the host immune response in the pathogenesis of SARS-CoV?
- What are the molecular targets for inhibiting coronavirus replication?

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