end of the RNA template for proper initiation of RNA synthesis and inhibits extension from a primed template [87].

4. HCV life cycle

4.1. Attachment and entry

Attachment of the virus to a cell followed by viral entry is the first step in the virus life cycle. In order to enter the host cell, the virus must first bind to a receptor on the cell surface. The specific interaction between a host cell receptor and viral attachment proteins on the surface of the virion determines tissue tropism and host range.

Low levels of HCV replication in cultured cells hindered study of the HCV life cycle. In order to overcome this, alternative models have been developed to study viral attachment and entry using recombinant HCV envelope proteins, including virus-like particles produced by baculovirus [88,89], vesicular stomatitis virus and retrovirus pseudotypes (HCVpp)[90–93], as well as infectious particles derived from a JFH-1 isolate (HCVcc) [94–96].

By using soluble E2 as a probe to identify cell-surface proteins potentially involved in HCV entry, CD81 was first identified as a putative HCV receptor [97]. CD81, a widely expressed 25-kDa cell surface protein, belongs to a family of tetraspanins and is involved in a number of activities, including cell adhesion, motility, metastasis, cell activation and signal transduction [98]. CD81 has a small and large extracellular loop, which mediate binding to recombinant E2 [97,99]. Studies with HCVpp and HCVcc confirm the involvement of CD81 in HCV entry. HCVpp shows a restricted tropism for human hepatic cell lines expressing CD81 [92,93,100–102]. Although necessary, CD81 expression alone is not sufficient for cell entry of HCVpp. Of note, HepG2, which does not express CD81 on its cell surface, is resistant to HCVpp infection, but over-expression of CD81 renders the HepG2 permissive to HCVpp infection [102–105]. Significant infection of CD81-negative cell lines with HCVpp has not been reported. However, as mentioned, not all CD81-positive cell lines can be infected [93,101,103]. Expression of CD81 in host cells is also required for infectivity of HCVcc. Recombinant CD81 and antibodies to CD81 have been observed to neutralize infection [94–96]. Thus, CD81 may function as a post-attachment entry co-receptor and may play a role after binding of the virion to another receptor.

The human scavenger receptor class B type I (SR–BI) has been identified as another putative receptor for HCV [106]. SR–BI is an 82-kDa glycoprotein with two C- and N-terminal cytoplasmic domains separated by a large extracellular domain involved in cellular lipometabolism. SR–BI is expressed in a wide variety of mammalian tissues and cell types [100,107,108], with particularly high levels of expression in the liver and steroidogenic tissue [107,109,110]. SR–BI recognition by soluble E2 requires the HVR1 of E2 [103,111]. A role of SR–BI in HCV cell entry has been confirmed using HCVpp in receptor competition assays using polyclonal anti-SR–BI serum, which has been observed to specifically inhibit HCVpp entry efficiently in a dose-dependent manner [103]. Recent reports have demonstrated that serum factors, especially high-density lipoprotein (HDL), a ligand to SR–BI, enhance the infectivity of HCVpp [105,112–115]. These results suggest that SR–BI modulates HCV entry.

Several human cell lines co-expressing CD81 and SR–BI are non-permissive for HCVpp infection [100,102,103], suggesting that another cell surface molecule(s) may be required for HCV entry. C-type (calcium-dependent) lectins, such as L-SIGN, DC-SIGN, and the asialoglycoprotein receptor, have also been investigated as potential HCV receptors based on their affinity for recombinant HCV envelope proteins [116–119]. However, L-SIGN and DC-SIGN are not expressed on hepatocytes and therefore cannot be receptors for HCV entry. A possible role of L-SIGN and DC-SIGN involves the capture and transfer of HCV to hepatocytes [120,121]. The LDL receptor is another candidate receptor based on the finding that HCV particles associate with lipoproteins in serum and their infectivity correlates with lipoprotein association. The LDL receptor has been shown to mediate HCV internalization by binding to virion-associated LDL particles [122]. However, a role for the LDL receptor in virus entry has not been confirmed using HCVpp [93], likely since the binding is mediated by lipoproteins rather than viral components.

Recently, a tight junction component claudin-1 has been identified as a co-receptor of HCV [123]. Claudin-1 appears to be critical for HCV entry into hepatic cells and is thought to act during the late stages of viral entry.

4.2. Translation

As opposed to cellular capped mRNA molecules which are translated via a cap-dependent scanning mechanism, the naturally uncapped RNA molecules of viruses such as flaviviruses and picornaviruses are translated via a cap-independent IRES-mediated process, in which viral protein expression is regulated by direct recruitment of each ribosome to the start site of translation [18,19]. The first 40 nt of the 5′UTR, which include a single stem–loop (domain I), are not essential for translation. Of note, the 5′ border of the IRES was mapped between nt 38 and 46 [17,124,125]. Other domains in the 5′UTR are more complex: domain II consisting of a stem with several internal loops, domain III consisting of a pseudoknot connected to a four-helix junction, as well as stem–loop IIId and domain IV, a small hairpin containing the AUG start codon at nt 342. It has been suggested that the first 12 to 40 nt downstream of the start codon are also important for IRES activity [126–128].

Structural analysis of the HCV IRES indicates that all of the RNA elements adopt tertiary structures capable of binding to the translation initiation complex with high affinity [129]. IRES-mediated translation of HCV RNA is initiated by direct binding of a vacant 40S ribosomal subunit to the IRES. The 40S subunit appears to interact with the viral RNA at multiple sites including stems, loops, pseudoknots, as well as the start codon. This binary complex then binds to eukaryotic initiation factor (eIF) 3, as well as the ternary complex eIF-2: Met–tRNA$_i$: GTP to form a 48S-like complex dependent upon both the basal domain III and the start codon. Subsequent formation of the 80S complex, which is the rate-limiting step, is dependent upon GTP

hydrolysis and attachment of the 60S subunit, after which the first peptide bond is formed [130].

In addition to the requirements described above, additional factors modulate IRES activity. Cellular factors such as the La autoantigen [131–133], heterogeneous ribonucleoprotein L [134], poly-C binding protein [135], and pyrimidine tract-binding protein (PTB)[136], have also been shown to bind to the IRES element and modulate HCV translation. HCV translation is also regulated through various interactions with viral proteins and the IRES.

We have found that HCV core protein expression inhibits HCV translation, possibly through binding to domain IIId, particularly a GGG triplet within the hairpin loop structure of the domain [137–139]. We therefore propose a model in which competitive binding of the core protein for the IRES and 40S subunit regulates HCV translation. Although there is an increasing body of evidence to suggest involvement of the core protein in translational regulation, there is conflicting data regarding the mechanism by which this occurs. In contrast to studies describing modulation of initiation of HCV translation by expression of the core protein [137,139–141], another study suggest that the core protein sequence, and not the core protein itself, modulates HCV IRES function through a long-range RNA-RNA interaction [142].

In addition to the10 known viral proteins (Fig. 1), the core-coding region of HCV has also been observed to express low levels of a 16–17 kDa protein [143–145]. This protein, which has been named the F protein, is thought to be produced by a+1 translational frameshift by ribosomes initiating translation at the start codon during synthesis of the HCV polyprotein, which shifts the reading frame between codons 9 and 11 of the polyprotein.

4.3. Polyprotein processing

The main translation product of the HCV genome is a large precursor polyprotein that is subsequently processed by cellular and viral proteases into mature structural and nonstructural proteins (Fig. 1). As deduced from the hydrophobicity profile and dependence on microsomal membranes, junctions at core/E1, E1/E2, E2/p7, p7/NS2 are processed by host signal peptidases. Secondary structure analysis of the core protein has revealed that all major alpha helices are located in the C-terminal half of the protein. A predicted alpha helix encoded by aa 174-191 is extremely hydrophobic and resembles typical signal peptide sequences. Further post-translational cleavage close to the C terminus of the core protein takes place, removing the E1 signal sequence by a signal peptide peptidase [146–149]. This peptidase has been identified as a presenilin-type aspartic protease [150] and shown to exhibit protease activity within cellular membranes, resulting in cleavage of peptide bonds in the plane of lipid bilayers.

As described above, HCV nonstructural proteins are processed by two viral proteases: cleavage between NS2 and NS3 is a rapid intramolecular reaction mediated by a NS2-3 protease spanning NS2 and the N-terminal domain of NS3, whereas the remaining four junctions are processed by a serine protease located within the 180 N-terminal residues of the NS3 protein. The NS3-NS5B region is presumably processed by sequential cleavage: NS3/4A→NS5A/5B→NS4A/4B→NS4B/5A [151–154]. Processing at the NS3/4A site is intramolecular, whereas cleavage at the other sites occurs intermolecularly.

4.4. RNA replication

As with other positive-strand RNA viruses, HCV replication is assumed to start with synthesis of a complementary negative-strand RNA using the genome as a template, after which genomic positive-strand RNA is produced from a negative-strand RNA template, both steps of which are catalyzed by the NS5B RdRp. The positive-strand RNA progeny are transcribed at a level 5- to 10-fold that of negative-strand RNA. Recombinant NS5B protein demonstrates RdRp activity in vitro, however, appears to lack strict template specificity and fidelity, which are essential for viral RNA synthesis. Thus, other viral and/or host factors are believed to be responsible for RNA replication and formation of the replication complex (RC), together with NS5B, which is required for catalyzing HCV RNA synthesis during replication. Several research groups have demonstrated HCV RC-mediated replication *in vitro* in crude membrane fractions of cells harboring subgenomic replicons [155–158]. Studies of cell-free replication systems, which provide a useful source of viral RCs have revealed that RNA synthesis can be initiated in the absence of additional negative-strand template RNA, suggesting that pre-initiated template RNA co-purifies with viral RCs [156–159].

Co-precipitation and immunostaining studies have revealed that newly synthesized HCV RNA exists as distinct specks of material, while all of the viral nonstructural proteins coexist [160]. These distinct structures may be equivalent to a membranous web, as described above. Expression of all structural and nonstructural proteins in the context of the entire HCV polyprotein has been observed to induce similar membrane changes [72]. It is of interest that morphologically similar structures, termed sponge-like inclusions [161], have been identified by electron microscopy within the hepatocytes of HCV-infected chimpanzees. Thus, HCV RC may exist in the context of a membranous web in infected cells. Because all nonstructural proteins of HCV are associated with the ER membrane in cells harboring subgenomic replicon RNA molecules [162,163], and since the membrane web is frequently observed in close proximity with the ER membrane, it is likely that the membranous web in HCV-infected cells is derived from the ER membrane.

On the other hand, there is accumulating evidence to support an association between HCV RNA replication and detergent-insoluble membrane domains or lipid rafts, which are microdomains rich in cholesterol and sphingolipids. Membrane flotation analysis and replication assays have shown that viral RNA and proteins exist within detergent-resistant, lipid-raft membranes, and that RNA replication occurs even after treatment with detergent [155,164]. Inhibitors of de novo sphingolipid synthesis have been shown to inhibit HCV replication, presumably by disrupting the association of viral nonstructural proteins with lipid rafts [165,166]. It is now accepted that HCV nonstructural proteins synthesized at the ER localize to lipid raft membranes when they are actively engaged in RNA replication.

Membrane separation analysis has demonstrated that HCV nonstructural proteins exist both in the ER and the Golgi apparatus, but that viral RNA replication primarily occurs in the Golgi fraction [155]. Further studies to elucidate the cellular processes involved in HCV RC formation and replication of the HCV genome in infected cells are needed.

Studies of RNA replicons have demonstrated the greatest viral RNA levels during the growth phase of the cells, after which a significant drop is observed as the cells reach confluence, suggesting that HCV replication and/or translation is tightly linked to host cell metabolism [163]. Huh-7 cells, in which adapted replicons are cured by treatment with IFN, yield cell populations that are more permissive for the replicon tested. Thus, it is likely that some interplay between the cellular environment and specific adaptive mutations of viral RNA contributes to efficient RNA replication of HCV.

Several cellular proteins capable of interacting with NS5A, such as vesicle-associated membrane protein-associated protein (VAP) subtypes A and B (VAP-A and –B)[73,167], FKBP8 [168], FBL2 [169,170], growth factor receptor-bound protein 2 adaptor protein [171], SRCAP [172], and karyopherin b3 [173], as well as Raf-1 kinase [174], have been identified. VAP-A and –B and SNARE-like proteins are known to localize within the ER and Golgi apparatus and are essential for HCV replication by binding with both NS5A and NS5B. VAP-A interacts with VAP-B through its transmembrane domain. Thus, VAP-A and -B are thought to be involved in the formation of functional HCV RCs. FKBP8, a member of the FK506-binding protein family, and Hsp90 form a complex with NS5A, further contributing to viral RNA replication. Statins that decrease the production of mevalonate by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase have been shown to inhibit HCV RNA replication [170,175], which can be reversed by adding geranylgeraniol, suggesting that viral replication requires geranylgeranylated proteins. A geranylgeranylated protein, FBL2, which contains an F-box motif and is therefore likely involved in protein degradation, has been identified as a NS5A-binding protein.

Host factors that interact with NS5B and might participate in HCV replication include cyclophilin B [176], p68 [177], nucleolin [178,179], and hnRNP A1 [180]. Cyclophilin B, a cellular peptidyl-prolyl cis-trans isomerase, interacts with the C-terminal region of NS5B to directly stimulate its RNA binding activity, and thereby contributes to efficient replication of HCV RNA. Redistribution of p68, an RNA helicase, from the nucleus to the cytoplasm occurs through its binding to NS5B, and the p68-NS5B interaction may further serve to mediate HCV replication. Nucleolin, a representative nulceolar marker, interacts with NS5B through two independent regions of NS5B and may be essential for HCV replication. hnRNP A1, a heterogeneous nuclear ribonucleoprotein, also interacts with septin 6, as well as the 5′-UTR and 3′-UTR of HCV RNA, and contains the cis-acting elements required for replication. Thus, hnRNP A1 and septin 6 play important roles in HCV replication through RNA-protein and protein-protein interactions. Other cellular components that bind to HCV RNA, such as PTB, may also be involved in viral replication. PTB has been observed to modulate HCV IRES activity by binding to several sites within the viral genome [22,181–184]. Recent studies have shown that PTB also forms part of the HCV RC and participates in viral RNA synthesis [185].

4.5. Viral assembly

Little is known about the assembly of HCV or its virion structure since efficient production of authentic HCV particles has only recently been achieved. As with related viruses, the mature HCV virion likely consists of a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. Various forms of HCV have been reported to circulate in the sera of infected hosts, including (i) free mature virions, (ii) virions bound to low-density lipoproteins and very-low-density lipoproteins, (iii) virions bound to immunoglobulins, as well as (iv) non-enveloped nucleocapsids, which exhibit different physicochemical and antigenic properties [186–189].

Several expression systems have been used to investigate HCV capsid assembly using lysate from mammalian cells, insects, yeast, bacteria, and reticulocytes, as well as purified recombinant protein [88,89,190–195]. The results suggest that the immunogenic nucleocapsid-like particles of HCV are variable in size ranging from 30 to 80 nm in diameter. The N-terminal half of the core protein is important for nucleocapsid assembly [190,194,195]. HCV capsid formation occurs in the presence or absence of ER-derived membrane, which supports cleavage of the signal peptide at the C-terminus [195].

Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. This process is thought to occur upon interaction of the core protein with viral RNA, and the core-RNA interaction may be critical for switching from RNA replication to packaging. In fact, HCV core protein can bind to positive-strand HCV RNA through stem–loop domains I, III and nt 24–41 [138]. Two-hybrid systems have identified a potential homotypic interaction domain within the N-terminal region of the core protein (aa 1–115 or –122), with particular emphasis on the region encompassing aa 82–102 [196,197]. Using purified HCV core protein, a C-terminally truncated core protein (aa 1–124) and structured RNA have been implicated in nucleocapsid formation to produce homogenous spherical HCV particles. When core protein containing the C terminus up to aa 174 is similarly examined, a heterogenous array of irregularly shaped particles is observed, suggesting that the C-terminus of the core protein influences self-assembly. Furthermore, Pro substitution within the C-terminal region has been observed to abolish core protein self-interaction [198]. Circular dichroism spectroscopy has further shown that a Trp-rich region spanning aa 76–113 is largely solvent-exposed and unlikely to play a role in multimerization. Recently, our group has demonstrated that self-oligomerization of the core protein is promoted by aa 72 to 91 of the core protein [49].

Once a HCV nucleocapsid is formed in the cytoplasm, it acquires an envelope as it buds through an intracellular membrane. Interactions between the core and E1/E2 proteins are thought to determine viral morphology. Expression of HCV structural proteins using recombinant virus vectors has succeeded in generating virus-like particles with similar ultrastructural

properties to HCV virions. Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the ER has been noted [88,199,200]. Mapping studies to determine the nature of interaction between core and E1 proteins have demonstrated the importance of C-terminal regions in this interaction [201,202]. Since corresponding sequences are not well conserved among various HCV isolates, interactions between core and E1 proteins might depend more on hydrophobicity than specific sequences. In contrast, it has been shown that the interaction between self-oligomerized HCV core protein and the E1 glycoprotein is mediated through a cytoplasmic loop of the polytopic form of the E1 protein [49].

It is believed that HCV particles are released from the cell through the secretory pathway. HCV structural proteins have been observed both in the ER and Golgi apparatus [203]. In addition, complex N-linked glycans, which transit through the Golgi apparatus, have been detected on the surface of HCV particles isolated from patient sera [204].

5. Perspectives

Since the discovery of HCV, which is a major cause of liver disease worldwide, significant progress has been made regarding the molecular biology of this virus. However, details regarding early and late stages of the HCV life cycle, including cell entry, genome packaging, assembly and release, remain unclear. In addition, the role of some viral proteins and their importance to replication remains unclear, as well as the role of certain host factors in regulation of the HCV life cycle.

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Brief Report

Enhancement of cytotoxicity against Vero E6 cells persistently infected with SARS-CoV by *Mycoplasma fermentans*

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Summary

We previously reported that cells with persistent severe acute respiratory syndrome coronavirus (SARS-CoV) infection were established after apoptotic events. In the present study, we investigated the cytopathic effects of dual infection with SARS-CoV and *Mycoplasma fermentans* on Vero E6 cells. Dual infection completely killed cells and prevented the establishment of persistent SARS-CoV infection. *M. fermentans* induced inhibition of cell proliferation, but the cells remained alive. Apoptosis was induced easily in *M. fermentans*-infected cells, indicating that they were primed for apoptosis. These results indicated that *M. fermentans* enhances apoptosis in surviving cells that have escaped from SARS-CoV-induced apoptosis.

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Severe acute respiratory syndrome (SARS) is a newly discovered infectious disease caused by SARS coronavirus (SARS-CoV), which became a global health threat due to its rapid transmission and high fatality rate [10, 20]. Vero E6 is a cell line derived from African green monkey kidney cells and is sensitive to SARS-CoV. SARS-CoV induces apoptosis into Vero E6 cells *via* activation of caspase-3 [14]. Activation of p38 mitogen-activated protein kinase (MAPK) induces cell death [14] and inactivation of Akt induces apoptosis [15]. In virus-infected cells, c-Jun N-terminal protein kinase (JNK), extracellular signal-related kinase (ERK)1/2, and 90-kDa ribosomal S6 kinases are also phosphorylated [13, 17]. Four groups, including our groups, independently reported that a small population of virus-infected cells remained alive after the

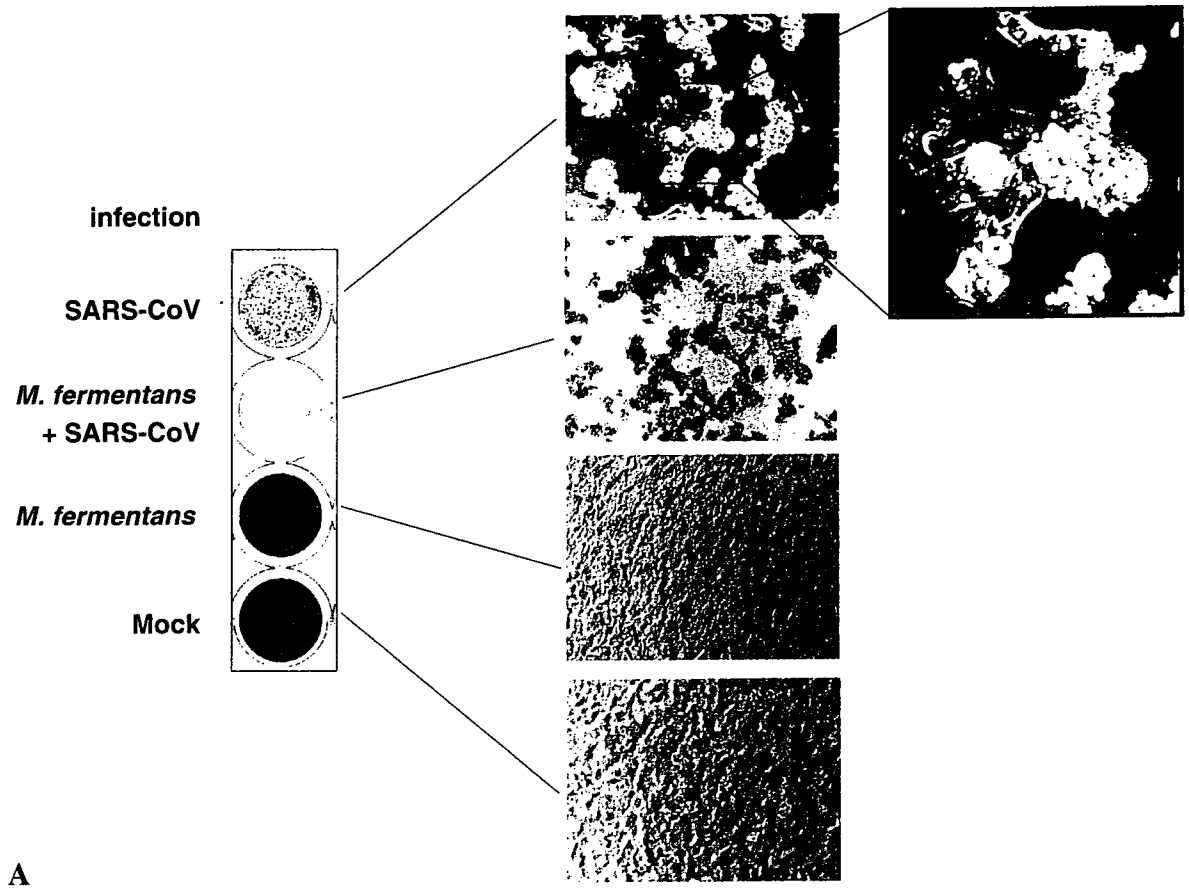
majority of virus-infected cells had died, and these cells grew with virus production [3, 16, 19, 24].

There have been reports of patients who were dual-infected with SARS-CoV and *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, or human metapneumovirus [2, 5, 9, 25]. However, the clinical significance of viral or bacterial co-infection with SARS in patients is still unclear. In the present study, we investigated cytopathic effects of *M. fermentans* on Vero E6 cells, and we investigated the cytotoxicity of dual infection with SARS-CoV and *M. fermentans* in Vero E6 cells. *M. fermentans* is known to enhance human immunodeficiency virus (HIV) replication [1]. Activation of NFκB by infection with *M. fermentans* increased replication of HIV by regulation of the long terminal repeat (LTR) [21, 23]. Thus, it is possible that *M. fermentans* can influence pathogenesis in co-infection with other viruses. *M. fermentans* is detected in approximately 10% of HIV-seronegative individuals [8], suggesting that a certain percentage of the healthy population is infected with *M. fermentans*. The present study was performed to examine whether pathogenicity is increased by co-infection with *M. fermentans* and SARS-CoV using an *in vitro* cell culture system.

Vero E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 0.2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) fetal bovine serum (FBS), and maintained at 37 °C in an atmosphere of 5% CO_2. In the present study, Vero E6 cells were treated at least three times with MC-210 (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), which is an antibiotic active against mycoplasma. After washing MC-210 from the medium, mycoplasma contamination was checked, and the cells were confirmed to be mycoplasma-free by incubation in glucose-containing PPLO medium. SARS-CoV, which was isolated as Frankfurt 1 and kindly provided by Dr. J. Ziebuhr, was used in the present study. Mycoplasma broth medium consisted of PPLO broth (Difco Laboratories, Franklin Lakes, NJ, USA), yeast extract (Difco), 15% heat-inactivated horse serum, 10% aqueous glucose, and 1000 units/ml penicillin as described previously [7]. In the present study, we used the *M. fermentans* M64 strain, which was isolated from a patient with acute respiratory disease. *M. fermentans* was grown aerobically in glucose-containing PPLO medium at 30 °C for 24 h, and then frozen at −80 °C.

Aliquots of 5×10^7 colony-forming units (CFU) of *M. fermentans* in 250 μl of glucose-containing PPLO medium were added to approximately 2×10^6 Vero E6 cells (100% confluency) in 1 ml of 5%-FBS-containing DMEM in 24-well plates. Mock-infected cultures were prepared with 250 μl of glucose-containing PPLO medium. After 24 h, the cells were infected with SARS-CoV at 2 m.o.i., and the cells were fixed and stained 7 days after virus infection. As shown in Fig. 1A, both *M. fermentans*- and mock-infected cells maintained confluency. No significant morphological changes were observed in the *M. fermentans*- or mock-infected cells when confluent cells were infected. The majority of SARS-CoV-infected cells died by apoptosis at 48 h.p.i., and persistently infected cells were observed at 7 days p.i. (Fig. 1A). Our recent studies indicated that these persistently infected cells grow well and produce viral particles in the medium [16]. On the other hand, all cells died 7 days after dual infection with *M. fermentans* and SARS-CoV. The observation in Fig. 1A raised questions regarding the stage at which *M. fermentans* kills cells that have escaped from apoptosis by SARS-CoV infection. We next examined the effects of *M. fermentans* infection at the late stages of SARS-CoV infection on cells persistently infected with SARS-CoV. Vero E6 cells were infected with SARS-CoV, and almost all cells died by apoptosis at 50 h.p.i. However, a small population of cells survived. At this time point, the cells were infected with *M. fermentans*. The cells were fixed and stained 8 days after SARS-CoV infection. As shown in Fig. 1B, persistently SARS-CoV-infected cells were not observed with additional *M. fermentans* infection. These results indicated that *M. fermentans* killed all surviving cells that had escaped from SARS-CoV-induced apoptosis and prevented the establishment of persistent SARS-CoV infection.

We investigated whether *M. fermentans* shows cytopathic effects on subconfluent Vero E6 cells. Approximately 5000 cells in 96-well plates were



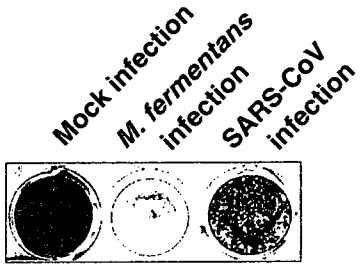


Fig. 1. Enhancement of cytotoxicity by dual infection with SARS-CoV and *M. fermentans*. **A** Confluent Vero E6 cells in 24-well plates were infected with 100 CFU/cell of *M. fermentans* for 24 h, and then the cells were infected with SARS-CoV at 2 m.o.i. A 20% volume of glucose-containing PPLO medium was added to all wells. After incubation for 7 days, surviving cells were observed in SARS-CoV-infected cultures, but not in dual-infected cultures. The cells were fixed with 10% formaldehyde for at least 24 h and stained with 0.1% naphthol blue black for 30 min. After washing with water, the plates were scanned using a GT-9400UF scanner (Epson, Tokyo, Japan). **B** Confluent Vero E6 cells were infected with SARS-CoV for 50 h, and then the cells were infected with *M. fermentans*. The cells were fixed and stained

inoculated with 5×10^6 CFU of *M. fermentans*. The results shown in Fig. 2A indicate that morphological changes in *M. fermentans*-infected cells were observed after day 1. The cells adopted an angular shape following infection with *M. fermentans*. As shown in Fig. 2B, cell growth was suppressed in the *M. fermentans*-infected cells. To clarify why cell proliferation is inhibited in *M. fermentans*-infected cells, Western blot analysis was performed using anti-retinoblastoma (Rb) antibody. Rb is thought

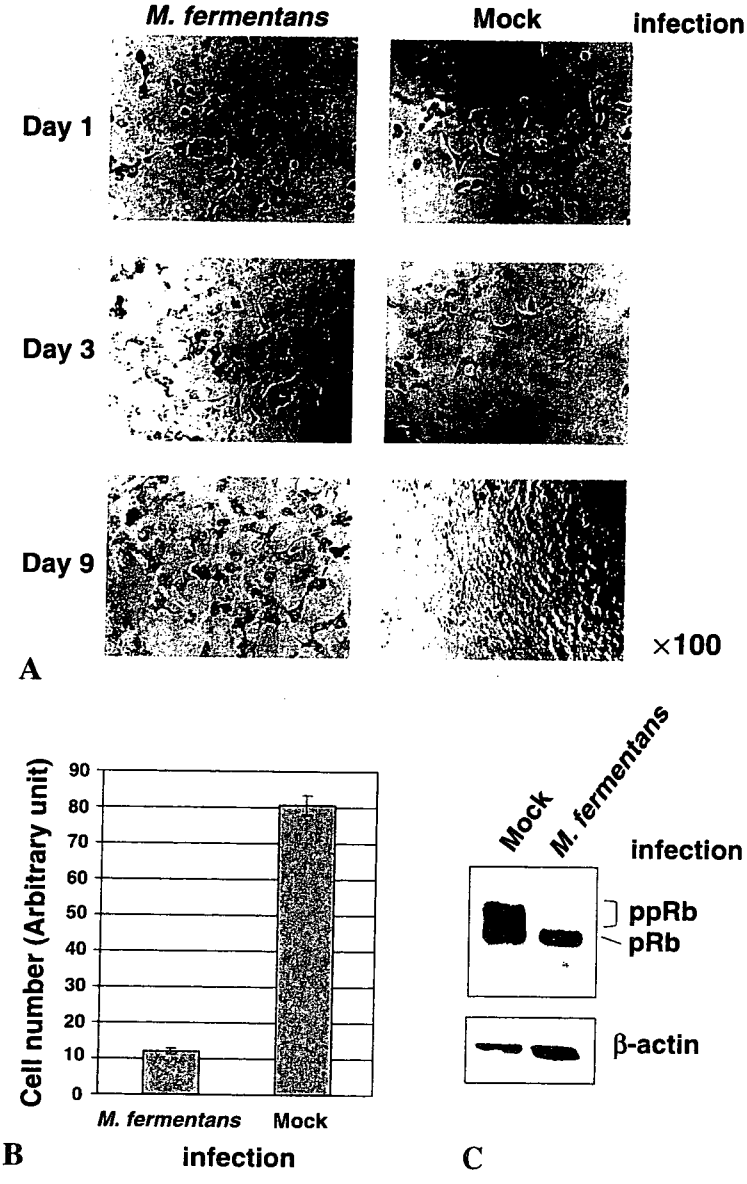


Fig. 2. Inhibition of cell proliferation by *M. fermentans* infection. **A** Subconfluent Vero E6 cells were infected with *M. fermentans* for 1, 3, and 9 days. **B** The cells at 9 days were fixed and stained. Cells were counted using a convenient method described by Everitt and Wohlfart for determination of the actual or relative number of cells in anchorage culture [4]. The dye-protein complexes were released hydrolytically with 0.1 M NaOH and measured spectrophotometrically at 660 nm. **C** Western blot analysis was performed using cell lysate 2 days after infection. Mouse anti-retinoblastoma protein (Rb) monoclonal antibody, which is able to detect the underphosphorylated form (pRb) and hyperphosphorylated from (ppRb), was purchased from BD Biosciences (Franklin Lakes, NJ, USA) and used at a dilution of 1:500. Mouse anti-β-actin antibody was purchased from Sigma and used at a dilution of 1:5000

to play one of key roles in the regulation of the G1 > S phase transition in the cell cycle, and phosphorylation of Rb is an important event in progression at G1 > S [18]. Only the hypophosphorylated form of pRb was detected in *M. fermentans*-infected cells at 2 days (Fig. 2C). The hypophosphorylated form of pRb is largely found in the early G1 phase.

Next, we examined the susceptibility of subconfluent *M. fermentans*-infected cells to apoptosis. Subconfluent cells were infected with *M. fermentans* for 24 h, and cycloheximide (final concentration, 1 mM) was added to the cells to stimulate apoptosis. As the cycloheximide was dissolved in DMSO, the same volume of DMSO alone was added to experimental controls. The subconfluent cells were infected with *M. fermentans* for 21 h, and cycloheximide or DMSO was added to the cells for 2 h. The cells were treated for 30 min with Apopercentage (Biocolor Ltd., Newtownabbey, Northern Ireland), which stains apoptotic cells at the early time stages by changing their color to red, and then the medium was replaced by PBS. As shown in Fig. 3A, the color of *M. fermentans*-infected cells treated with

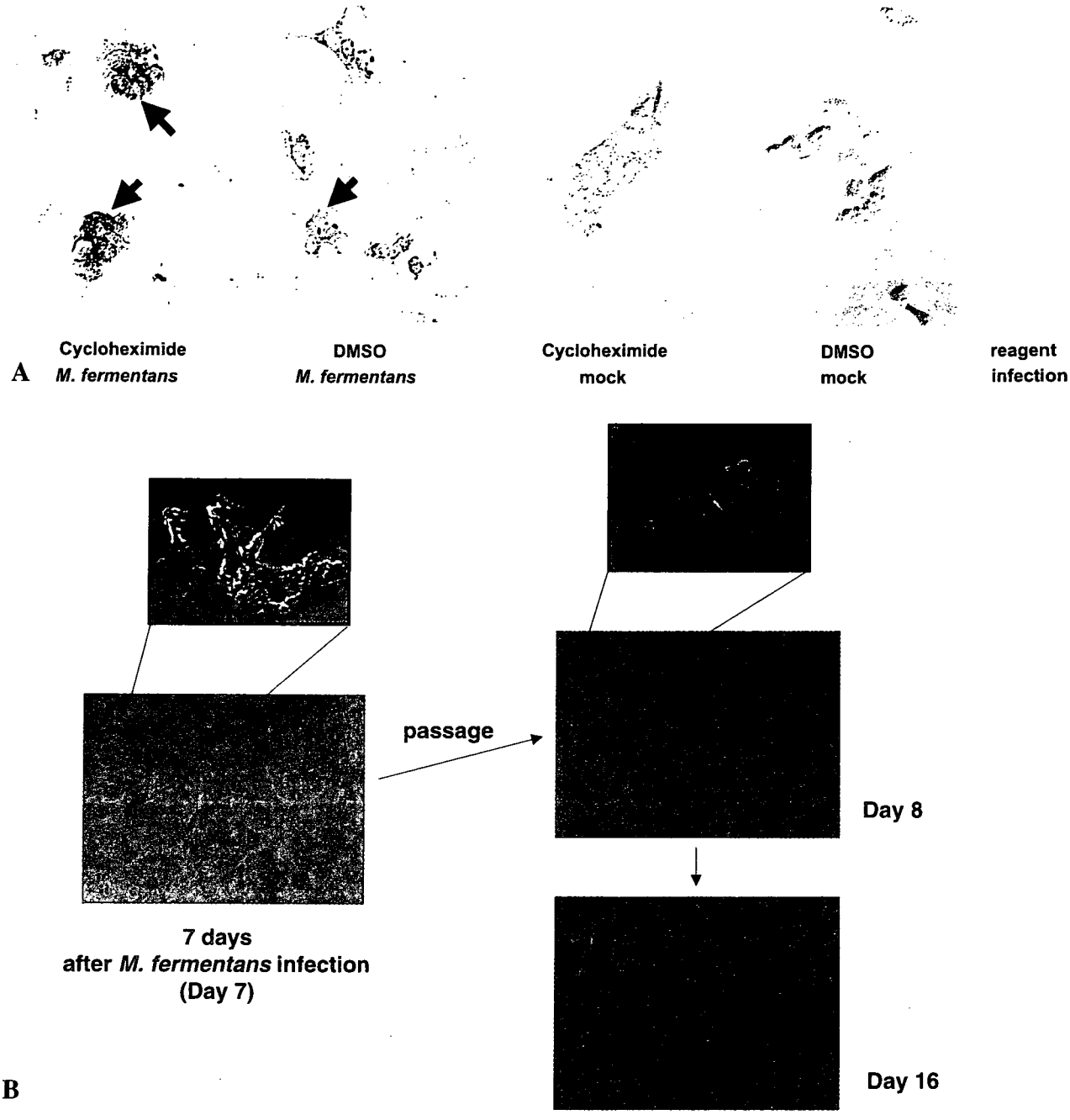


Fig. 3. Viability of *M. fermentans*-infected cells. **A** Subconfluent cells were infected with *M. fermentans* for 21 h, and then the cells were treated with cycloheximide. Apopercentage was used for detection of apoptosis. The arrows indicate apoptotic cells. **B** After 7 days postinfection, cells were passaged and cultured for 1 and 9 days

cycloheximide changed to red, indicating apoptosis, whereas mock-infected cells showed no change in color. However, some DMSO-treated cells infected with *M. fermentans* also changed color to red, but DMSO-treated cells without infection did not, suggesting that stimulation with not only cycloheximide but also DMSO induced apoptosis of *M. fermentans*-infected cells. Therefore, this result

indicated that *M. fermentans*-infected cells were primed for apoptosis. It remained to be determined whether *M. fermentans*-infected cells were alive. Therefore, subconfluent *M. fermentans*-infected cells at 7 days were trypsinized and resuspended in 5%-FBS-containing DMEM with MC-210 to kill the mycoplasma. After one day, almost all cells were attached to the plate, and dividing cells were observed (Fig. 3B). This result indicated that the *M. fermentans*-infected cells were alive and were able to grow when *M. fermentans* was removed.

In the present study, we demonstrated enhancement of cytotoxicity against Vero E6 cells persistently infected with SARS-CoV by *M. fermentans*. As *M. fermentans* processes phospholipase C in the cell membrane [22], the morphological changes caused by infection of Vero E6 cells with *M. fermentans* may be due to partial destruction of cell-surface lipids. In addition, cell death by superinfection of persistently SARS-CoV-infected cells with *M. fermentans* may be induced by phosphorylated p38 MAPK [12, 14], and assembly of the SARS-CoV envelope on the cell surface in surviving cells may also be a trigger of cell death on infection with *M. fermentans*. Akt, JNK, Bcl-2 and Bcl-xL play important roles in the establishment of SARS-CoV-persistent infection [12], and nucleocapsid (N) protein of SARS-CoV using a vaccinia virus expression system [6] is able to induce phosphorylation of Akt and JNK, but not p38 MAPK [12]. Glycogen synthase kinase 3β, which is downstream of Akt, was phosphorylated in N-expressing cells (data not shown). These results suggested that the N protein plays important roles for preventing apoptosis. On the other hand, Vero E6 cells were primed for apoptosis by *M. fermentans* infection in our experimental system, but cell death was not induced by infection. As infection by an excess of *M. fermentans* more than used in this study sometimes kills subconfluent Vero E6 cells, *M. fermentans* itself may be able to kill Vero E6 cells. Therefore, when the number of surviving cells that have escaped from cell death by SARS-CoV infection is very low, it is thought that cell death is enhanced by apoptotic effects of both SARS-CoV and *M. fermentans* infection.

The results of this study demonstrate that cells stressed by infection with *M. fermentans* are subject to further stress after infection with SARS-CoV. This phenomenon is important for understanding clinical pathogenicity, because it is unlikely that patients will be infected with only a single pathogen. There have been no previous reports regarding the pathological implications of dual infection with SARS-CoV and viruses or bacteria using a cell culture system. The findings of the present study have implications for infection control of acute or persistent SARS. Dual infection of the kidney cell line Vero E6 with SARS-CoV and *M. fermentans* also provides important information to further our understanding of renal infection in SARS patients.

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