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Identification of an Alternative 5′-Untranslated Exon and New Polymorphisms of Angiotensin-Converting Enzyme 2 Gene: Lack of Association With SARS in the Vietnamese Population

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We analyzed genetic variations of angiotensin-converting enzyme 2 (ACE2), considering that it might influence patients' susceptibility to severe acute respiratory syndrome-associated coronavirus (SARS-CoV) or development of SARS as a functional receptor. By cloning of the full-length cDNA of the *ACE2* gene in the lung, where replication occurs on SARS-CoV, it was shown that there are different splicing sites. All exons including the new alternative exon, exon-intron boundaries, and the corresponding 5′-flanking region of the gene were investigated and 19 single nucleotide polymorphisms (SNPs) were found. Out of these, 13 SNPs including one non-synonymous substitution and three 3′-UTR polymorphisms were newly identified. A case control study involving 44 SARS cases, 16 anti-SARS-CoV antibody-positive contacts, 87 antibody-negative contacts, and 50 non-contacts in Vietnam, failed to obtain any evidence that the *ACE2* gene polymorphisms are involved in the disease process in the population. Nevertheless, identification of new 5′-untranslated exon and new SNPs is considered helpful in investigating regulation of ACE2 gene expression in the future. © 2005 Wiley-Liss, Inc.

KEY WORDS: angiotensin-converting enzyme 2 (ACE2); severe acute respiratory syndrome (SARS); SARS associated coronavirus (SARS Co-V); virus receptor; polymorphism; association study

INTRODUCTION

Severe acute respiratory syndrome (SARS) is an emerging infectious disease characterized by systemic inflammation followed by atypical pneumonia [Peiris et al., 2003b]. Shortly after the initial worldwide outbreak in 2003, SARS-associated coronavirus (SARS-CoV) was discovered as an etiological agent of SARS [Drosten et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Peiris et al., 2003a], and then angiotensin-converting enzyme 2 (ACE2) was identified as a functional receptor of this newly arrived virus [Li et al., 2003]. More recently, CD209L was reported as being another alternative receptor for the virus, but it appears to be a less efficient entry site than ACE2 [Jeffers et al., 2004].

Virus receptors generally play a key role in the entry of the pathogen into the host cells and may influence development or progression of viral diseases. For example, it is well known that genetic polymorphism of chemokine receptor 5 (CCR5), a co-receptor for human immunodeficiency virus-1 (HIV-1), influences the natural history of HIV-1 infection. The mutant allele CCR5-Δ32 does not produce a functional protein and has been shown to protect host cells against HIV-1 infection, and progression into acquired immunodeficiency syndrome is delayed after seroconversion takes place [Dean et al., 1996; Liu et al., 1996; Samson et al., 1996]. By analogy with the above, we considered that genetic polymorphisms of ACE2 could influence SARS-CoV infection or clinical manifestations of SARS.

ACE2 is a homologue of ACE1 and exhibits 40% identity of amino acid sequence to its N- and C-terminal domains [Tipnis et al., 2000]. Similar to ACE1, ACE2 is a metalloprotease that constitutes a renin-angiotensin system. Human full-length ACE2 cDNAs have been cloned already from lymphoma (GenBank accession No. AF241254) [Tipnis et al., 2000], cardiac left ventricle (AF291820) [Donoghue et al., 2000] and testis (AY623811) [Douglas et al., 2004]. Based on published data, it has been said that the ACE2 gene (*ACE2*) contains 18 exons, and spans approximately 40 kb of genomic DNA on the human X-chromosome. Although *ACE2* mRNA expressions were demonstrated in the lung by the method of quantitative reverse transcription-PCR (RT/PCR) [Harmer et al., 2002] and its protein expression was obviously shown by immunohistochemistry [Hamming et al., 2004], full-length *ACE2* cDNA has not been cloned from the lung so far. This is considered to be

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very likely as being an important replication site of SARS-CoV [Haagmans et al., 2004].

In the present study, we attempted a full-length cloning of *ACE2* cDNA from the human lung and found a new alternative, the 5′-untranslated exon. During this process, an extended region of the original exon 1 was identified in the testis' RNAs. Then, we explored genetic polymorphisms within 19 exons including new regions and the 5′-flanking region of *ACE2* and tried to determine whether the polymorphisms of *ACE2* are associated with SARS in Vietnamese.

MATERIALS AND METHODS

Cloning of ACE2 cDNA From the Lung

Cloning was performed by combination of RT/PCR and 5′- and 3′- rapid amplification of cDNA ends (RACE) procedures, using human lung total RNA (Stratagene, La Jolla, CA) and human testis total RNA (Stratagene) as a control. The total RNAs were reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT)$_{12-18}$, and then cDNA was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) with primers ACE2-exon 1s (5′-CAA AGG CTG ATA AGA GAG AA-3′) and ACE2-exon 18 as (5′-GAA CAG AAG TCA AAT CCA GA-3′) to amplify the transcript of 2721 bp encompassing the original 18 exons of *ACE2* gene on database.

The First Choice RLM-RACE Kit (Ambion, Austin, TX) was used for 5′- and 3′-RACE procedures following the manufacturer's recommendation. Gene-specific primer sets for 5′-RACE were ACE2-5′Outer1 and ACE2-5′Inner1 (5′-GTG GAT ACA TTT GGG CAA GT-3′ and 5′-CCT AGA CTA AAA CCT CCT CA-3′), and ACE2-5′Outer2 and ACE2-5′Inner2 (5′-GAA GTA AGA AAG CCT CCA CA-3′ and 5′-CTC CTG ATC CTC TGT AGC CA-3′). Gene specific primer set for 3′-RACE was ACE2-3′Outer and ACE2-3′Inner (5′-CAA TGA TGC TTT CCG TCT GA-3′ and 5′-ACA CTT GGA CCT CCT AAC CA-3′). Nucleotide sequences of PCR products were directly determined by the automated DNA sequencer (PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA).

To investigate expression of the exons on the 5′ side, RT/PCR procedures were performed on the total RNAs of human lung, testis, trachea (Stratagene), primary-cultured bronchial epithelial cells [Lechner and LaVeck, 1985], small intestine (Ambion), and on the human major organ cDNAs (Bio Chain Institute) with the sense primer New-exon (5′-TTC TTA CTT CCA CGT GAC CT-3′) or Extended-exon 1 (5′-GCT CAG CAG ATT GTT TAC TG-3′) and the antisense primer ACE2-5′Outer1.

Genomic DNA Samples for the Association Study

An association study between SARS patients and controls was reviewed and approved by local ethics committees. Of 62 cases fulfilling the World Health Organization case definition of probable SARS in Vietnam [WHO, 2003], 5 fatal cases and 3 non-Vietnamese cases were excluded from this study. In the remaining 54 cases, 44 individuals agreed to participate in this study as cases. One hundred and three Vietnamese staff members, who did not develop SARS but may have come in contact with SARS patients in the hospital where nosocomial infection of SARS had arisen, were enrolled as contacts. Furthermore, 50 medical staff members who had been working in a separate building and those considered having no history of contact with SARS patients joined in this study as non-contacts, according to information obtained by questionnaire. Peripheral blood samples of all the subjects were collected and genomic DNA was extracted from the blood cells by a method described elsewhere [Wang et al., 1994].

Testing for Antibody Response to the SARS-CoV

To detect the antibody to the SARS-CoV in serum, all the blood samples were tested with SARS ELISA (Genelabs Diagnostics Pte. Ltd., Singapore Science Park, Singapore) in accordance with the manufacturer's recommendation [Guan et al., 2004].

Identification of Polymorphisms Within *ACE2* Gene

Of the 44 SARS cases and 103 contacts recruited, a half of the samples were randomly selected for searching polymorphisms within the *ACE2* gene. PCR primers were designed to amplify 19 exons including the new alternative exon, exon-intron boundaries and approximately 1,000 bp of the 5′-flanking region of the new exon, reaching 2,000 bp upstream of the 5′-end of the original exon 1 (Table I). Genomic DNA of each sample was subjected to PCR amplification followed by direct sequencing.

Genotyping of Identified Polymorphisms

Non-synonymous nucleotide substitutions and other variations with a minor allele frequency higher than 0.05 were subjected to genotyping in all SARS cases, contacts and non-contacts. Consequently, one novel non-synonymous substitution, two possible non-synonymous polymorphisms in the database (dbSNP identification nos. rs4646116 and rs11798104), and variations of 3′-UTR in exon 18 (position 39844) and of intron 3 (rs2285666, position 8789) were genotyped by the combination of direct sequencing method and single-strand conformation polymorphism (SSCP) analysis or PCR-based restriction fragment length polymorphism (RFLP) analysis.

Statistical Analysis

Disease associations were assessed by the chi-square test. The P values less than 0.05 were considered significant in all the tests and data analysis was carried out using JMP version 5 (SAS Institute, Inc., Cary, NC).

RESULTS

Full-Length ACE2 cDNAs From the Lung and Expression of the Transcripts

By the use of the RT/PCR encompassing all known exons of *ACE2* and 3′-RACE method, we could amplify *ACE2* cDNA as PCR fragments completely corresponding to the published sequence of *ACE2* cDNA (AF241254). The 5′-RACE procedure on the total RNA of the lung demonstrated the presence of a new alternative exon (registered as AB193259), which consisted of a segment between position −1141 and −942 and was connected to the 5′-end of the original exon 1. The 5′-end of transcripts was extended to position −1141 repeatedly by both sets of gene-specific primers. In addition, novel 65 nucleotides on the 5′-side (registered as AB193260), extending the 5′-end of the original exon 1 upstream, were amplified from the total RNA of testis. A schematic diagram of the exon-intron structure is shown in Figure 1.

RT-PCR revealed that the expression of the new alternative exon could be seen not only in the lung but also in the testis, trachea, bronchial epithelial cells, small intestine, and various major organs (data not shown). The new extended region was expressed not only in the testis but also in other organs including bronchial epithelial cells and the small intestine (data not shown).

TABLE I. Primers Used to Identify Polymorphisms Within the *ACE2* Gene

Region	Primer name	Primer sequence (5′–3′)	Product size
5′ flanking region	ACE2-pro-1-sense	TAA TTC AGT CAG TGC TTG C	676 bp
	ACE2-pro-1-anti	AAT AGT GGA GGC ATA GAT AAA	
5′ flanking region	ACE2-pro-2-sense	TTT GTG AGC TGC TTT ATT TT	618 bp
	ACE2-pro-2-anti	TGC CAG AGT GTA TGT ATG AG	
New alternate exon	ACE2-new-sense	TTA TTG CAA TGT CAC CTG A	470 bp
	ACE2-new-anti	TTA TGA CTA CTC TCC ACT CCA	
5′ flanking region	ACE2-pro-3-sense	TTT GAA TAG GTA AGT GAA GG	669 bp
	ACE2-pro-3-anti	TAG AAC TAG GGA TCA TGA AGA	
5′ flanking region	ACE2-pro-4-sense	TGA ATT CCA TAA AGA CAA GG	653 bp
	ACE2-pro-4-anti	AAA CTT GTC CAA AAA TGT CTT	
Exon 1	ACE2-ex1-sense	ATC TTT AAC AGC TTT CTA GGA	644 bp
	ACE2-ex1-anti	AAC ATC CAA TCT CAC AAC TC	
Exon 2	ACE2-ex2-sense	AAC TCA TCT ATG TCA CAG CAC	636 bp
	ACE2-ex2-anti	AAA TTA TAT GGA CAC CTT ACC	
Exon 3	ACE2-ex3-sense	ACT TCT TTG GGT TTT GGT AG	627 bp
	ACE2-ex3-anti	ACA TCA GGT CAT AAA GTG GTT	
Exon 4	ACE2-ex4-sense	TCA TTT CAG TGG TTT ATT TTC	521 bp
	ACE2-ex4-anti	CTT TTC TTT TTC CCC AGT A	
Exon 5	ACE2-ex5-sense	CTT GTA TGG TTC TTG TGC TT	535 bp
	ACE2-ex5-anti	GGG CTG TCC TAT TAT TCT CTA	
Exon 6	ACE2-ex6-sense	ACC TGT GTT CTC CCA AGT A	568 bp
	ACE2-ex6-anti	CTT TAT CAT TTG AAT TGC AG	
Exon 7	ACE2-ex7-sense	TCA CCA AGT TAA GTA CAC GAA	562 bp
	ACE2-ex7-anti	TAC ACC TGC AAT TCA AGT TAT	
Exon 8	ACE2-ex8-1-sense	TTG CAG TGA GAA CAT TTG AAA	560 bp
	ACE2-ex8-1-anti	CCT CTG TTG TCT CCC ATT T	
Exon 8	ACE2-ex8-2-sense	GCT GTG CAG TAG ATC TCA AA	643 bp
	ACE2-ex8-2-anti	CAG ATT GTC CAC AGG TTC A	
Exon 9	ACE2-ex9-sense	CTA TGA GCA AGA GAA CAG G	577 bp
	ACE2-ex9-anti	TCA CCA GTA GTA ATT TCC AGT	
Exon 10	ACE2-ex10-sense	AGG GAG GAA ACT GAA ACT AAT	587 bp
	ACE2-ex10-anti	GGT ATC CAA ATG GAG ACT AAA	
Exon 11	ACE2-ex11-sense	GTG CAC ACC TAT AAA CCA AG	615 bp
	ACE2-ex11-anti	TGA GCA TGT TTA GGG TAG AC	
Exon 12	ACE2-ex12-sense	GTG AAA GGG CTA TTA ATC TGT	612 bp
	ACE2-ex12-anti	GAG AGG GCT GTA GTT ATG A	
Exon 13	ACE2-ex13-sense	CAG GAA CCT AGA CCA TAC AA	636 bp
	ACE2-ex13-anti	GTT GCT TTC ACT ATG TCT CA	
Exon 14	ACE2-ex14-sense	GTA CAA ATT AGG TCA TGG C	550 bp
	ACE2-ex14-anti	GAC GAG AGT CAA TTG AAA G	
Exon 15	ACE2-ex15-sense	ATT ATT GGG TTT CAT CTC G	637 bp
	ACE2-ex15-anti	TAT AGG TCA ATG AAG GCA G	
Exon 16	ACE2-ex16-sense	CAG AAC AAA TAG TGC CAA A	610 bp
	ACE2-ex16-anti	CAT AGT GGT AAC TTG CTT GAT	
Exon 17	ACE2-ex17-sense	GCT CTG TCA CCT AGG CTA G	633 bp
	ACE2-ex17-anti	CTA GGA AGA TGA ACT GCT GAT	
Exon 18	ACE2-ex18-1-sense	TTA AGA TGA ATC CTA GCA GTG	655 bp
	ACE2-ex18-1-anti	CAT TTA GAT TAT CCC TGA ACA	
Exon 18	ACE2-ex18-2-sense	TCT GGA TTT GAC TTC TGT TC	623 bp
	ACE2-ex18-2-anti	AAC ACT GTG AGC AAA TAC AAA	
Exon 18	ACE2-ex18-3-sense	GAA CAG GTA GAG GAC ATT G	531 bp
	ACE2-ex18-3-anti	GGG TAG TGA CTG TGA GAA ATA	

Subgrouping of Subjects Based on the Status of Anti-SARS-CoV Antibody

Basic characteristics and sub-grouping of subjects are shown in Table II. The 44 SARS cases, 103 contacts, and 50 non-contacts were analyzed in the present study. Based on anti-SARS-CoV antibody titer in serum, the contacts were further divided into two subgroups, antibody-positive contacts, and antibody-negative contacts (data not shown).

Identification of Polymorphisms Within *ACE2* Gene

All exons including the new exon, exon-intron boundaries and the corresponding 5′-flanking region of *ACE2* were tested

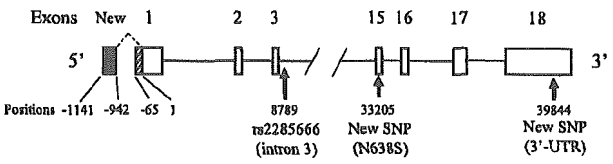

Fig. 1. A schematic diagram of the *ACE2* gene structure and the positions of SNPs. The known exons are depicted as open boxes. A solid box and a striped box indicate the new exon and the new extended region of the exon 1, respectively. The arrows represent locations of the SNPs analyzed in a case-control study. The broken line depicts an alternative-splicing site.

TABLE II. Demographic Findings of Subjects and Subgroups

Groups	SARS cases (n = 44)	Contacts (n = 103)	Anti-SARS-CoV antibody		Non-contacts (n = 50)
			Positive (n = 16)	Negative (n = 87)	
Age (years), mean [range]	39.3 [17–76]	36.5 [15–68]	36.0 [25–50]	36.6 [15–68]	—[a]
Male/female	13/31	46/57	7/9	39/48	17/33

[a]Data not available.

to identify variations of *ACE2* among SARS cases and contacts. As shown in Table III, 19 single nucleotide polymorphisms (SNPs) were identified. Six of them have already registered on dbSNP database, and 13 SNPs including one non-synonymous substitution, from asparagine to serine at 638 (N638S) in the exon 15 (position 33205) were identified. All SNPs but one in intron 3 (rs2285666, position 8789) and another in exon 18 (position 39844) were found to be considerably rare among both SARS cases and contacts tested. In subsequent analysis, we therefore chose polymorphisms, and analyzed possible non-synonymous substitution, excluding rare non-coding variants among SARS patients and contacts.

Genotype and Allele Frequency of Three SNPs

Two SNPs in intron 3 and exon 18 with minor allele frequencies higher than 0.05 and a newly identified non-synonymous SNP, N638S in exon 15 were analyzed in all samples (Table IV). Relative positions of these SNPs are shown in Figure 1. Genotyping results by direct sequencing method were confirmed by RFLP or SSCP methods. Because *ACE2* is located to the X chromosome in humans, samples from both males and females were analyzed, respectively. Two possible non-synonymous SNPs that are shown in the dbSNP database (rs4646116 and rs11798104) were not found in our samples this time. When the antibody-negative contacts group was compared with antibody-positive group including SARS cases in either males or females, no difference was observed between the two groups both in regards to genotype and allele frequencies. Comparison between antibody-positive contacts and SARS cases, and comparison between contacts and non-contacts did not show any significant differences in genotype and allele frequencies of the tested polymorphisms.

DISCUSSION

During the worldwide outbreak of SARS in 2003, a subset (about 20%–30%) of SARS patients required mechanical ventilation, having developed pneumonia. The fatality rate was 11%, although the majority of patients recovered without unfavorable outcome [Peiris et al., 2003b]. As a natural consequence, asymptomatic individuals produce antibodies against SARS-CoV in their sera [Ip et al., 2004; Woo et al., 2004]. In one of the studies, it was shown that 2.3% of contacts who did not develop clinical SARS had serum antibody titer over the threshold [Ip et al., 2004], and this implies the presence of asymptomatic individuals.

We hypothesized that the functional polymorphism of ACE2, which is considered as being a virus receptor of SARS-CoV, might influence the clinical history of SARS-CoV infection at least in part. This is because, a variation of the co-receptor to HIV, CCR5-Δ32 where allele frequency is approximately 10% in the European population [Martinson et al., 1997], has been well known to resist HIV infection and alter its clinical course [Dean et al., 1996; Liu et al., 1996; Samson et al., 1996].

TABLE III. SNPs Within the *ACE2* Gene

Region	Position[a]	dbSNP rs# cluster ID	Change of nucleotide (major/minor allele)	Change of amino acid (major/minor allele)	No. of individuals who had the minor allele	
					SARS cases	Contacts
5′ flanking region	−751	NEW[b]	C/T	—	1	1
5′ flanking region	−671	NEW	G/A	—	1	1
5′ flanking region	−634	NEW	C/G	—	1	0
Intron 3	***8789***	***rs2285666***	***A/G***	—	***15***	***32[c]***
Intron 6	13286	rs4646140	G/A	—	0	1
Intron 9	25082	NEW	G/A	—	0	1
Intron 10	25424	NEW	G/A	—	0	1
Intron 10	27418	rs4646165	G/A	—	0	1
Intron 12	28946	rs2301693	C/T	—	0	2
Intron 12	29018	rs2301692	A/G	—	0	2
Intron 14	30816	NEW	A/G	—	1	1
Intron 14	30867	rs4646174	C/G	—	0	2
Intron 14	33121	NEW	G/C	—	1	0
Exon 15	33205	NEW	A/G	N/S	0	1
Intron 16	36655	NEW	G/A	—	0	1
Intron17	38926	NEW	C/T	—	0	1
Exon 18 (3′-UTR)	39663	NEW	C/G	—	0	1
Exon 18 (3′-UTR)	39705	NEW	A/G	—	0	1
Exon 18 (3′-UTR)	***39844***	***NEW***	***G/A***	—	***3***	***4[c]***
					No. of samples tested = 20	No. of samples tested = 57

[a]Position numbers indicate distance from 5′ end of the original exon 1.
[b]Newly identified SNPs are shown as NEW.
[c]Minor allele frequencies of the SNPs shown in bold and italic were higher than 0.05.

TABLE IV. Genotype and Allele Distribution of Three Single Nucleotide Polymorphisms (SNPs)

					Contacts		
				SARS cases	Antibody (+)	Antibody (−)	Non-contacts
Intron 3 (rs2285666)							
	Male	Genotype/allele[a] no. (frequency)	A	5 (0.38)	4 (0.57)	21 (0.54)	5 (0.31)
			G	8 (0.62)	3 (0.43)	18 (0.46)	11 (0.69)
			Total no.	13	7	39	16
	Female	Genotype no. (frequency)	A/A	12 (0.39)	4 (0.44)	15 (0.31)	11 (0.33)
			A/G	16 (0.51)	3 (0.33)	24 (0.50)	17 (0.52)
			G/G	3 (0.10)	2 (0.22)	9 (0.19)	5 (0.15)
			Total no.	31	9	48	33
		Allele no. (frequency)	A	40 (0.65)	11 (0.61)	54 (0.56)	39 (0.59)
			G	22 (0.35)	7 (0.39)	42 (0.44)	27 (0.41)
Exon 15 (N638S)							
	Male	Genotype/allele no. (frequency)	A	13 (1.00)	7 (1.00)	39 (1.00)	17 (1.00)
			G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
			Total no.	13	7	39	17
	Female	Genotype no. (frequency)	A/A	31 (1.00)	8 (0.89)	47 (0.98)	33 (1.00)
			A/G	0 (0.00)	1 (0.11)	1 (0.02)	0 (0.00)
			G/G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
			Total no.	31	9	48	33
		Allele no. (frequency)	A	62 (1.00)	17 (0.94)	95 (0.99)	66 (1.00)
			G	0 (0.00)	1 (0.06)	1 (0.01)	0 (0.00)
Exon 18 (3′-UTR)							
	Male	Genotype/allele no. (frequency)	G	12 (0.92)	7 (1.00)	37 (0.95)	17 (1.00)
			A	1 (0.08)	0 (0.00)	2 (0.05)	0 (0.00)
			Total no.	13	7	39	17
	Female	Genotype no. (frequency)	G/G	27 (0.87)	8 (0.89)	46 (0.96)	29 (0.88)
			A/G	4 (0.13)	1 (0.11)	2 (0.04)	4 (0.12)
			A/A	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
			Total no.	31	9	48	33
		Allele no. (frequency)	G	58 (0.94)	17 (0.94)	94 (0.98)	62 (0.94)
			A	4 (0.06)	1 (0.06)	2 (0.02)	4 (0.06)

[a]Genotype distribution is the same as allele distribution in male.

Using the PCR-based cloning procedure, we identified for the first time an alternative exon upstream of the original exon 1 of *ACE2* that is expressed in various organs, including the lung and trachea, primary-cultured bronchial epithelial cells, and the small intestine. These are considered to be important replication sites of SARS-CoV [Haagmans et al., 2004]. Both 5′- and 3′-ends of the intron between the new alternative exon and the original exon 1 followed the GT/AG rule of Breathnach and Chambon [1981]. Although the organ specificity of the transcripts was not confirmed in this study due to the limitation of non-quantitative PCR amplification, implication of the new exon was definitely shown in the lung and small intestine. Also, we found the extended region of the original exon 1, 65 bp on the 5′ side. Neither the new alternative exon nor the new extended region of exon 1 gave rise to a new coding region and they were considered as 5′-untranslated region.

It was recently reported that genetic variations of ACE2 did not affect SARS susceptibility or outcome in Hong Kong [Chiu et al., 2004]. In that study, five intronic SNPs (rs2106809, rs2285666, rs4646142, rs714205, and rs2074192) were chosen and analyzed in a case-control manner, based on the previously known exon-intron structure and SNPs already registered in the database. By contrast, we attempted to analyze not only previously known SNPs but also variations newly identified among actual SARS patients and contacts. Based on the information from the exon-intron structure of *ACE2* cloned by ourselves, we searched for nucleotide sequences in all the exons including the new alternative exon and the corresponding 5′-flanking region, which are thought to contain promoters of the new exon and the original exon 1. We found one novel non-synonymous substitution N638S and 18 non-coding SNPs including two relatively common SNPs with minor allele frequency higher than 5%. We selected these SNPs and analyzed them furthermore in a case-control manner, because, while they are rare occurrence, non-synonymous substitution may directly modulate the function of the protein, and because relatively common SNPs can often be used as markers to ascertain a causative variation. Of 19 SNPs found in this study, 13 were new polymorphisms, 3 of which were located in 3′-UTR. Two possible non-synonymous SNPs in dbSNP database were not found in the population tested. Judging from the results so far obtained in this case-control study, there was no statistical evidence that ACE2 polymorphisms affect SARS infection or alter its clinical course. However, type II error was not negligible because of a relatively small size of samples tested.

Taking also into consideration, the results from a previous study of *ACE2* polymorphisms by others [Chiu et al., 2004], it is unlikely that the genetic defect of ACE2 is involved in the disease resistance that has been shown in CCR5-Δ32 in HIV-1 infection cases. Nevertheless, this newly identified alternative 5′-untranslated exon expressed in the lung, and also newly recognized polymorphisms in this study might be of great help concerning investigations into the regulation of *ACE2* gene expression and the possible significance of the variations in further more in-depth studies.

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Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection

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A unique coronavirus severe acute respiratory syndrome-coronavirus (SARS-CoV) was revealed to be a causative agent of a life-threatening SARS. Although this virus grows in a variety of tissues that express its receptor, the mechanism of the severe respiratory illness caused by this virus is not well understood. Here, we report a possible mechanism for the extensive damage seen in the major target organs for this disease. A recent study of the cell entry mechanism of SARS-CoV reveals that it takes an endosomal pathway. We found that proteases such as trypsin and thermolysin enabled SARS-CoV adsorbed onto the cell surface to enter cells directly from that site. This finding shows that SARS-CoV has the potential to take two distinct pathways for cell entry, depending on the presence of proteases in the environment. Moreover, the protease-mediated entry facilitated a 100- to 1,000-fold higher efficient infection than did the endosomal pathway used in the absence of proteases. These results suggest that the proteases produced in the lungs by inflammatory cells are responsible for high multiplication of SARS-CoV, which results in severe lung tissue damage. Likewise, elastase, a major protease produced in the lungs during inflammation, also enhanced SARS-CoV infection in cultured cells.

cell entry | protease | spike protein | SARS

Severe acute respiratory syndrome (SARS) is caused by a SARS-associated coronavirus (SARS-CoV), a newly emergent member in a family of Coronaviridae (1–6). Unlike other human coronaviruses, SARS-CoV causes a fatal respiratory disease in humans (1–6). Coronavirus is an enveloped virus with a positive-stranded large genomic RNA with ≈30 kb (7). Spikes exist on the virion surface and resemble solar corona, each of which is composed of a trimer of the spike (S) protein (7, 8). The S protein is a type I fusion protein of an approximate molecular weight of 180 kDa. The prototypical coronavirus mouse hepatitis virus enters into cells via the cell surface, although a variant isolated from persistent infection enters from an endosome, the low pH of which induces its fusion activity (9). However, the entry pathway of SARS-CoV appears to be distinct from that of the other coronaviruses. Simmons *et al.* (10) hypothesized that SARS-CoV enters cells by an endosomal pathway, and S protein is activated for fusion by trypsin-like protease in an acidic environment. This idea is based on the following two findings: (*i*) SARS-CoV infection can be blocked by lysosomotropic agents, and (*ii*) S protein expressed on cells is activated for fusion by trypsin. These results were obtained by studies using pseudotype retroviruses harboring SARS-CoV S protein on the envelope and those using S protein expressed on cells by expression vectors (10).

In the present study, we show that various proteases, as well as trypsin, are effective in inducing the fusion of SARS-CoV-infected VeroE6 cells. These proteases facilitated SARS-CoV entry from the cell surface, which indicates that SARS-CoV has the potential to enter cells via two different pathways, either an endosomal or a nonendosomal pathway, depending on the presence of proteases. More interestingly, SARS-CoV entry from cell surface mediated by protease resulted in >100-fold more efficient infection than entry through endosome. Elastase, a major protease produced during lung inflammation, also manifested this enhancing effect. These findings suggest that severe illness in the lungs and intestines is attributable to the proteases produced in these organs during an inflammatory response or in the presence of certain physiological conditions.

Materials and Methods

Cells and Viruses. VeroE6 cells were grown in DMEM (Nissui, Tokyo), supplemented with 5% FBS (GIBCO/BRL). The SARS-CoV Frankfurt 1 strain, kindly provided by J. Ziebuhr (University of Würzburg, Würzburg, Germany) (1), was propagated and assayed by using Vero E6 cells.

Proteases. Various proteases were dissolved in PBS (pH 7.2) and used at the indicated concentrations in DMEM containing 5% FCS. The proteases used in this study were trypsin (Sigma, T-8802), thermolysin (Sigma, P 1512), chymotrypsin (Sigma, C-3142), dispase (Roche, 1 276 921), papain (Worthington, 53J6521), proteinase K (Wako, Tokyo), collagenase (Sigma, C-5183), and elastase (Sigma, E-0258).

Plaque Assay. VeroE6 cells prepared in 24-well plates were inoculated with 50 μl of 10-fold serially diluted virus samples and incubated at 37°C for 1 h. Cells were then cultured with 0.5 ml per well of DMEM containing 1% FCS and 0.75% methyl cellulose (Sigma) for 2 d. Cells were fixed with 1 ml of 10% formaldehyde per well for at least 2 h. After removing the culture fluids, cells were irradiated overnight under a UV lamp and stained with crystal violet. Plaques produced by SARS-CoV were counted under light microscopy. Titration was done in duplicate and infectivity was displayed by plaque-forming units (pfu).

Western Blotting. S protein expressed in Vero E6 cells was analyzed by Western blotting. Preparation of cell lysates, SDS/PAGE, and electrical transfer of the protein onto a transfer membrane were described (11). S protein was detected with anti-S Ab, IMG-557 (Imgenex, San Diego) and horseradish peroxidase-conjugated anti-rabbit IgG Ab (anti-R-IgG, ALI3404, BioSource International, Camarillo, CA). The bands were visualized by using enhanced chemiluminescence reagents (ECL-plus, Amersham Pharmacia) on a LAS-1000 instrument (Fuji).

Real-Time PCR. VeroE6 cells in 96-well culture plates were treated with DMEM containing 1 μM bafilomycin (Baf; Sigma, B-1793) and 5% FCS (DMEM plus Baf) at 37°C for 30 min and then chilled on ice for 10 min. Approximately 10^4 pfu of virus in DMEM plus Baf were infected to 10^4 cells on ice; multiplicity of

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Abbreviations: SARS-CoV, severe acute respiratory syndrome-coronavirus; S, spike; pfu, plaque-forming unit; moi, multiplicity of infection; Baf, bafilomycin.

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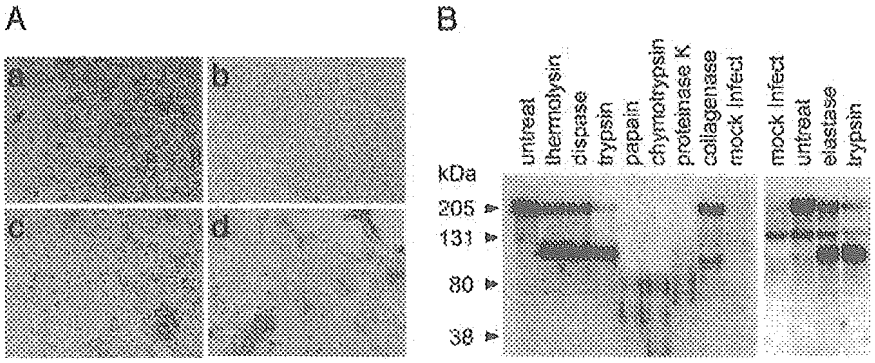

Fig. 1. Induction of cell-fusion and SARS-CoV S protein cleavage by proteases. (*A*) Syncytium formation after treatment with trypsin. VeroE6 cells cultured in 24-well plates were infected (*b* and *d*) or mock-infected (*a* and *c*) with the SARS-CoV Frankfurt 1 strain at moi = 0.5 and incubated at 37°C for 20 h. Cells were washed once with PBS and treated (*c* and *d*) or untreated (*a* and *b*) with 200 μg/ml trypsin for 5 min. Those cells were cultured for a further 4 h and observed by microscopy. (*B*) Western blot analysis of S protein treated with various proteases. Cells infected as described above were treated either with thermolysin (200 μg/ml), dispase (1 unit/ml), trypsin (200 μg/ml), papain (0.74 unit/ml), chymotrypsin (1 mg/ml), proteinase K (8 μg/ml) collagenase (200 μg/ml), or elastase (1 mg/ml), as described above. Soon after treatment, cells were lysed with lysing buffer, and S protein was analyzed by Western blot after SDS/PAGE. To detect the S protein (S2 fragment), mAb IMG-557 was used at a concentration of 5 μg/ml.

infection (moi) was at 1. After 30-min adsorption, the virus was removed, and infected cells were treated for 5 min with various concentrations of proteases in DMEM plus Baf that was prewarmed at room temperature. After protease was removed, cells were cultured in DMEM plus Baf at 37°C for 6 h. Vero E6 cell monolayers in 24-well plates were infected with 10 pfu of SARS-CoV (moi = 0.0001). After 30-min adsorption, cells were cultured in DMEM containing 5% FCS in the presence or in the absence of various proteases for 20 h. To isolate cellular RNA, 100 and 500 μl of isogen (Nippon Gene, Toyama, Japan) were added to each well of 96- and 24-well plates, respectively, together with 5 μg of yeast RNA as a carrier for 2-propanol precipitation. RNA was prepared according to the manufacturer's instructions and finally dissolved in 20 μl of diethyl pyrocarbonate-treated water. Real-time PCR was performed to estimate the amounts of mRNA9 in a final volume of 20 μl of 1× LightCycler RNA Master Mix (Roche Diagnostics) by using the RNA isolated as described above. For amplification of the fragment from mRNA9, we used 500 nM of a pair of oligonucleotides 5′-CTCGATCTCTTGTAGATCTG-3′ (SARS leader) and 5′-TCTAAGTTCCTCCTTGCCAT-3′ (SARS mRNA9 reverse). Amplified DNA from mRNA has 240 bases. With these primers, genomic RNA was not detected because the fragment to be amplified from genomic RNA would be ≈30 kb. For detection by hybridization, 200 nM each of the hybridization probes 5′-ACCAGAATGGAGGACGCAATGGGGCAAG-3′ (3′FITC labeled), 5′-CCAAAACAGCGCCGACCCCAAG-GTTTAC-3′ (5′LCRed640 labeled) were used. PCR analysis was performed under the following conditions [reverse transcription: 61°C, 20 min; PCR, 95°C, 30 s (95°C, 5 s; 55°C, 15 s; 72°C, 10 s) ×45 cycles] with a LightCycler instrument (Roche Diagnostics). To measure the amounts of viruses that entered into cells, we infected cells with 10-fold stepwise diluted SARS-CoV from 10^6 to 10^2 pfu, and the amounts of mRNA9 were determined by real-time PCR. The amounts of virus that entered into cells after protease treatment were calculated from a calibration line obtained as above and shown as relative mRNA levels. When relative mRNA9 was higher than 10^6 pfu, samples were diluted and reexamined so that they were placed between 10^6 and 10^2 pfu.

Results

Activation of Cell Fusion and SARS-CoV S Protein Cleavage by Various Proteases. VeroE6 cells susceptible to SARS-CoV were infected with the Frankfurt-1 strain of SARS-CoV at a moi of 0.5, and those infected cells were treated with trypsin at 20 h after infection. Cell fusion was detected from 2 h after trypsin treatment (Fig. 1*Ad*). Fusion was also found after treatment with thermolysin or dispase (data not shown). Little or no fusion occurred after treatment with papain, chymotrypsin, proteinase K, or collagenase. S proteins in cells treated with proteases that induce fusion were cleaved approximately in the middle (Fig. 1*B*), a finding similar to that of Simmons *et al.* (10). In contrast, no apparent S2 band was detected in cells bearing S proteins treated with proteases that failed to induce fusion (Fig. 1*B*). These results showed that various proteases, including trypsin, activate the fusion activity of the SARS-CoV S protein by inducing its cleavage. Further, SARS-CoV infection was extensively inhibited by treatment of cells with Baf (Fig. 2*A*, no Baf vs. Baf without protease). These results suggest that SARS-CoV takes an endosomal pathway for its entry, and that S protein cleavage is important for fusogenicity, which is consistent with the conclusions of a previous report (10).

SARS-CoV Entry from Cell Surface Facilitated by Proteases. If the hypothesis proposed by Simmons *et al.* (10) is correct, we can make SARS-CoV enter cells directly from their surface by attaching the virus there and treating them with trypsin and other proteases that induce fusion. Treatment of VeroE6 cells with Baf at a concentration of 1 μM suppressed SARS-CoV infection via the endosomal pathway to <1/100, as shown in Fig. 2*A*. The cells treated with Baf were inoculated with SARS-CoV at a moi of 1 and incubated on ice for 30 min (adsorption on ice does not allow virus to enter cells). Then cells were treated with various proteases for 5 min at room temperature and incubated at 37°C for 6 h. Virus entry was estimated by the newly synthesized mRNA9 measured quantitatively by real-time PCR. A calibration curve of real-time PCR (Fig. 2*C*), showing the level of mRNA9 after infection with 10-fold diluted SARS-CoV, was used to estimate the amount of infected virus from the mRNA levels. As shown in Fig. 2*A*, thermolysin and trypsin, two proteases with fusion-inducing activity, extensively facilitated viral entry. In contrast, two proteases that did not induce fusion, papain and collagenase failed to do so. Treatment of cells with trypsin before virus infection did not facilitate viral entry (Fig. 2*B*), indicating that effects of trypsin on cells are not involved in this infection. Other proteases did not influence the SARS-CoV infection as trypsin, when treated before virus inoculation (data not shown). Protease treatment of SARS-CoV before infection did not enhance infectivity but reduced it by 10- to 100-fold (data

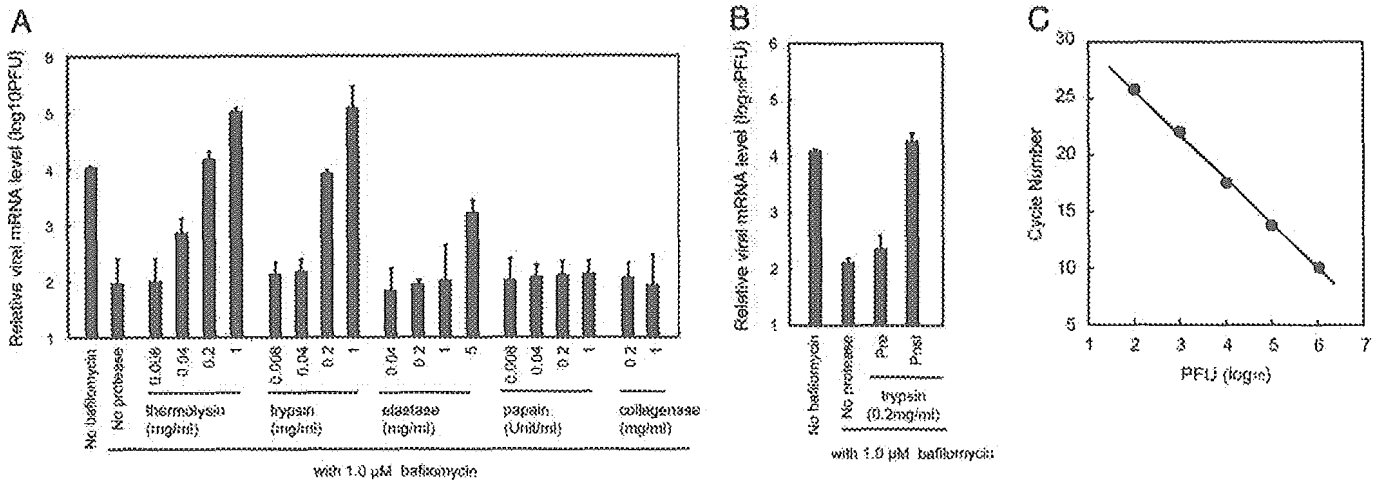

Fig. 2. Entry of SARS-CoV from cell surface facilitated by proteases. (*A*) Effect of proteases on SARS-CoV entry into VeroE6 cells treated with Baf. VeroE6 cells in 96-well plates were treated with Baf at a concentration of 1 μM at 37°C for 30 min, placed on ice and infected with SARS-CoV at moi = 1 for 30 min. Then, cells were treated with various concentrations of different proteases at room temperature for 5 min and cultured in the presence of Baf for a further 6 h. The amount of mRNA9 was measured quantitatively by real-time PCR. Cells untreated with Baf or those treated with Baf but untreated with protease were used as controls. The relative viral mRNA level is displayed by virus infectivity (pfu) calculated from a calibration line shown in C. (*B*) Cells treated with Baf at 37°C for 30 min were then treated with trypsin at room temperature for 5 min before (pre) or after (post) virus inoculation, and virus infection was estimated quantitatively by real-time PCR as described above. (*C*) Calibration in real-time PCR. VeroE6 cells in 94-well plates were infected with 10-fold step diluted viruses, and mRNA9 levels at 6 h after infection were estimated by real-time PCR. The relationship is shown between inoculated pfu (*x* axis) and cycles of real-time PCR to reach a positive level (amount of mRNA9) (*y* axis).

not shown). We believe these results demonstrate that SARS-CoV, when adsorbed onto the cell surface, fuse with the plasma membrane of its envelope with S protein, which is cleaved into S1 and S2 by proteases with fusion-inducing activity. This suggests a nonendosomal, direct entry of SARS-CoV into cells in the presence of proteases. Those findings also support the hypothesis drawn by Simmons *et al.* (10) that trypsin-like protease plays an important role in facilitating membrane fusion.

Enhancement of SARS-CoV Infection by Various Proteases. Treatment with a high concentration of thermolysin and trypsin augmented virus entry or replication by 10-fold or higher, as compared with the standard infection (Fig. 2*A*, e.g., compare bar 6 or bar 10 with bar 1 from the left). We then compared the replication kinetics of SARS-CoV in cells treated with Baf and a high concentration of trypsin with that of cells maintained without Baf or trypsin. The level of mRNA9 was always ≈10-fold higher in trypsin-treated cells at any given time during the early period of infection (Fig. 3). These data also imply that viral replication after entry via the cell surface proceeds ≈1 h ahead of that via the endosomal pathway, suggesting that the surface route is more efficient for rapid viral replication.

Because SARS-CoV replication was shown to be enhanced by trypsin treatment, we next assessed the efficiency of virus spread in the presence or absence of trypsin in a low moi, which mimics natural infection in target organs. Virus (10 pfu) were inoculated onto 10^5 confluent VeroE6 cells (moi = 0.0001), and the cells were incubated at 37°C for 20 h in the media with or without trypsin. The level of mRNA9 estimated quantitatively by real-time RT-PCR showed that virus replication was 100- to 1,000-fold higher when cells were cultured in the presence of trypsin, when compared with replication in the absence of trypsin (Fig. 4*A*). Viral infectivity of the supernatants in SARS-CoV-infected cells cultured with or without trypsin also indicated that trypsin treatment enhanced viral growth by ≈100-fold (Fig. 4*B*). We also examined growth kinetics of SARS-CoV in the presence of low-concentration proteases (62.5 μg/ml trypsin, 125 μg/ml elastase) that do not detach cells from plates during culture for 42 h. It was also shown that protease enhanced virus replication (Fig. 4*C*) with remarkable fusion formation (Fig. 4*D*). All of these results strongly suggest that the virus spreads efficiently from cell to cell in the presence of trypsin, which cleaves S to S1 and S2 to allow cell entry of SARS-CoV via the cell surface.

We next examined the effects on low moi by other proteases that facilitate SARS-CoV entry from VeroE6 cell surface. As shown in Fig. 5, all of the proteases that produce S2 (Fig. 1*B*) and that induce cell-cell fusion enhanced virus spread. In contrast, those proteases that did not generate S2 and that did not induce cell-cell fusion failed to enhance the infection. These observations suggest that proteases that facilitate SARS-CoV entry from the cell surface support efficient SARS-CoV infection. Thus, protease is likely to be responsible for the high multiplication of

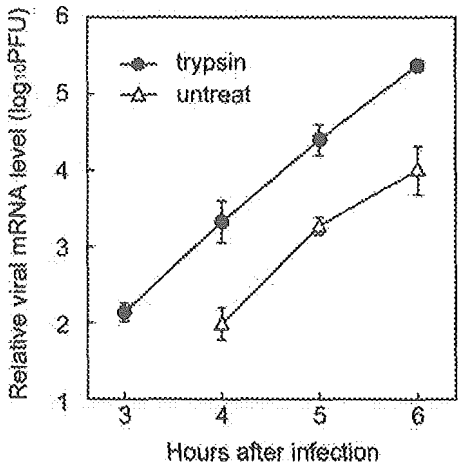

Fig. 3. Kinetics of mRNA9 synthesis after treatment of trypsin. VeroE6 cells were treated with Baf, infected with SARS-CoV, and treated with 200 μg/ml trypsin as described in the legend to Fig. 2*A*. The amount of mRNA9 synthesized was monitored by real-time PCR at 3–6 h after inoculation. VeroE6 cells without any treatment were also infected as a control (untreated). Relative viral mRNA level is displayed by virus infectivity (pfu) calculated from the calibration line shown in Fig. 2C.

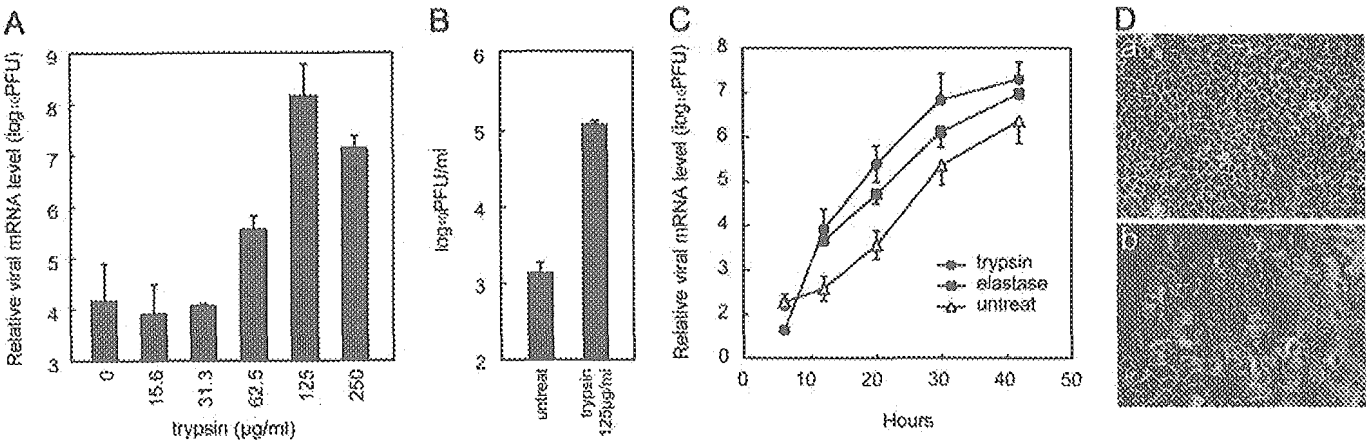

Fig. 4. Enhancement of SARS-CoV infection by proteases. (*A*) Effect of trypsin on virus replication in VeroE6 cells. Approximately 1×10^5 VeroE6 cells cultured in 24-well plates were infected with 10 pfu of SARS-CoV (moi = 0.0001) and cultured in the presence of varied trypsin concentrations. Viral replication was estimated at 20 h after infection by the amount of mRNA9, as measured by real-time PCR. (*B*) Viral infectivity was examined by plaque assay after 20-h incubation in the presence or absence of trypsin (125 μg/ml). (*C*) Viral growth kinetics after infection was examined in cultures in the presence or absence of trypsin (62.5 μg/ml) or elastase (125 μg/ml) by real-time PCR. Cells were harvested from 4 to 42 h after infection at intervals and the level of mRNA9 was monitored. Relative viral mRNA level is displayed by virus infectivity (pfu) calculated from a calibration line (*A–C*). (*D*) Cytopathic changes of virus-infected cells cultured in the presence (*b*) or absence (*a*) of trypsin (125 μg/ml) for 42 h are shown.

SARS-CoV in the major target organs of SARS, such as the lungs and bronchus, where various proteases are produced (e.g., by inflammatory cells), as well as in the intestines, where a number of proteases are physiologically secreted.

One of the major proteases produced by inflammatory cells in the lungs is an elastase produced by neutrophils (12), the accumulation of which was reported in the lungs of SARS patients (13). The level of elastase in bronchoalveolar lavage fluids was reported to reach levels as high as 700 μg/ml (12). Accordingly, we determined whether this protease has the potential to enhance SARS-CoV infection in a fashion similar to that of trypsin or thermolysin. Elastase was revealed to enhance SARS-CoV infection in cultured VeroE6 cells in terms of S protein cleavage (Fig. 1*B*), its cell-surface-mediated entry pathway (Fig. 2*A*), and its growth enhancement ability after low moi (Figs. 4*C* and 5). These results strongly suggest that SARS-CoV replication can be enhanced in the lungs by elastase.

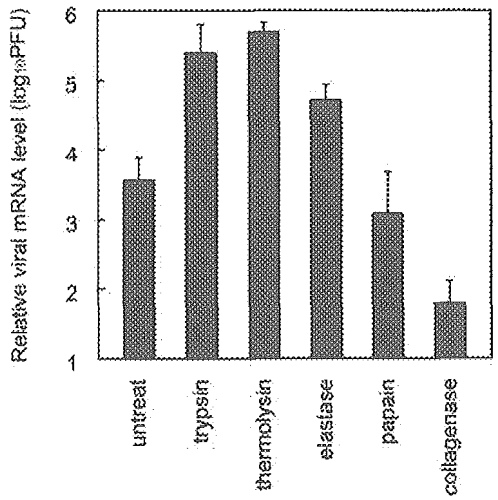

Fig. 5. Effect of various proteases on virus replication in VeroE6 cells. VeroE6 cells in 24-well plates were infected as described in Fig. 4 and cultured in the presence of trypsin (62.5 μg/ml), thermolysin (12.5 μg/ml), elastase (125 μg/ml), papain (0.037 unit/ml), or collagenase (200 μg/ml). At 20 h after infection, the amounts of mRNA9 were measured by real-time PCR. Relative viral mRNA level is displayed by virus infectivity (pfu) calculated from the calibration line.

Discussion

The SARS-CoV gene and viral antigens were found in a number of organs, such as the liver, cerebrum, pancreas, and kidneys, as well as in such major target organs as the bronchus, lungs, and intestines (14–17), with the latter showing drastic tissue damage by SARS-CoV infection, whereas the other organs were not so severely affected. Although the pathogenic mechanism of SARS has not been elucidated, the present study suggests that proteases secreted in major target organs play an important role in the high multiplication of virus in those organs, which, in turn, results in severe tissue damage. An initial infection by SARS-CoV in pneumocytes via its receptor ACE2 (18), the endosomal pathway, could induce inflammation that generates a variety of proteases such as elastase. Once those proteases are present in the lungs, they may mediate an ensuing robust infection, which may result in enhanced replication of SARS-CoV in the lungs. Although lung damage is postulated to be mediated by cytokines by a so-called cytokine storm (14, 16), higher virus multiplication could also contribute to the cytokine storm by killing a large number of infected cells. A variety of proteases secreted in the small intestines, another major target organ of SARS-CoV, could also be responsible for the high growth of SARS-CoV in these tissues, which could result in a high rate of diarrhea in SARS patients (19, 20).

Protease-mediated enhancement of infection is known for orthomyxovirus and paramyxovirus infections (21–24), in which their envelope glycoprotein is not fully cleaved in *de novo* synthesized cells, and thus the virus particles produced contain partially cleaved or uncleaved glycoprotein. Those glycoproteins on virions are cleaved after treatment with protease, which results in the enhancement of infectivity. Thus, trypsin affects directly virons and increases the infectivity of those viruses. However, enhancement of SARS-CoV infection by trypsin or other proteases is mediated by another mechanism. Although trypsin treatment *in vitro* induces cleavage of the S protein on virions, such treatment never enhances SARS-CoV infectivity but reduces it to 1/10–1/100 of the original titer. Only S protein bound to its receptor ACE2 and cleaved by proteases could obtain fusion activity. Based on this idea, it is most likely that

binding of S protein to ACE2 induces conformational changes of the S, which is inevitable to be correctly processed for fusion activity by proteases. In other words, proteases can successfully induce the fusion activity of S protein only after S-ACE2 binding. Alternatively, protease treatment of virions digests out the S1 portion important for ACE2 binding, resulting in a loss of infectivity, whereas S2 alone is sufficient for fusion after binding to its receptor despite loss of the S1 fragment.

Why is the infection via the endosomal pathway not as efficient as direct infection from the cell surface? Throughout our examinations, replication deriving from the cell surface pathway began 1 h ahead of that via the endosomal pathway. We assume that a virus needs ≈1 h for trafficking from the cell surface where virion binds to ACE2 to the endosome. When cells are infected with an extremely low moi, a condition that occurs in natural infection, a 100- to 1,000-fold higher rate of infection was observed in the presence of proteases. Thus a 10-fold difference at 6 h after inoculation could result in a 1,000-fold difference, provided that one cycle of SARS-CoV replication is ≈6 h (25) and three rounds of infection take place within 20 h.

The present studies suggest that coinfection of SARS-CoV with some other non- or low-pathogenic respiratory agents, such as *Chlamydia*, mycoplasma, or bacteria, results in severe lung disease, which is attributed to the proteases produced by the infection with those non-SARS-CoV agents, as has been shown by the enhancement of respiratory diseases caused by influenza virus coinfected with nonpathogenic bacteria (26, 27). Studies are in progress to see whether coinfection exacerbates pneumonia in mice infected with SARS-CoV.

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