(Table 1). Contd.....

Amino Acids	Function	References
141-149	HA-binding site	[23]
145-148	Heparin binding site	[18]
146-149	Nuclear localization signal motif	[35]
149, 166, 167	Heparin binding site	[139]
189, 191	Glycosaminoglycans/polyamions	[16]
189-194	HA-binding site	[23]
212, 214	Glycosaminoglycans binding site	[16]
227	Protein kinase A phosphorylation site Anti-angiogenic activity Neutrophic activity	[59, 137]
255-258, 290, 291, 296, 299, 300	Collagen-I binding site	[17, 18, 22]
285	Secretion activity	[140]
272-279	Cytotoxic T-lymphocyte activity	[138]
354-359	Cell differentiation activity Neuroprotective activity	[94, 141, 142]
373-380, 376, 377	Secretion activity	[11]
387-411	Anti-invation activity Anti-VEGF expression activity	[55]
389-397	Cytotoxic T-lymphocyte activity	[138]
415-418	Secretion activity	[11]

cells, and a structural change in PEDF is thought to be a mechanism for enhanced receptor binding [12].

PEDF binds to collagen type I and type III, but not collagen type II and type IV [21]. An increase in ionic strength, lower pH, or modifications of carboxylic groups of PEDF decrease the affinity of PEDF for collagen I, suggesting that acidic and /or negatively charged sites of PEDF (Glu41, Glu42, Glu43, Asp44, Asp64, Asp256, Asp258, Glu290, Glu291, Glu296, Asp300, and Glu304) are possible collagen I-binding sites [22]. Collagen I-binding sites are also localized to the side opposite the heparin-binding site. At this site, the acidic amino acid residues Asp255, Asp257, and Asp299 are critical to collagen I-binding [18]. Mutation of the collagen I-binding site (aa299) of PEDF abolishes anti-tumor activity through anti-angiogenic activity [17].

PEDF is found within hyaluronan (HA) rich tissues and contains amino acids sequence for putative HA-binding motifs, BXBX2BX2B and BX3AB2XB motifs (B represents basic amino acids: X represents residues excluding acidic amino acids: A represents negatively charged amino acids). Becerra *et al.* examined the HA-binding site of PEDF by site-directed mutagenesis and identified two HA-binding motifs (aa141-149 and aa189-194) in PEDF [23]. The BXBX2BX2B motif (aa189-194) was located between α-helix F and β-strand s3A and the BX3AB2XB motif (aa141-149) was localized between β-strand s2A and α-helix F. Although PEDF is a member of the serine-protease inhibitor (serpin) superfamily, none of the other serpins have these HA-binding sites [23].

PEDF RECEPTOR-BINDING SITES

PEDF is a secreted protein with various biological effects and deposits in the cell membrane. In addition, effects of PEDF are blocked by antibodies which are cell surface-binding antagonists [24-27]. These findings suggest an interaction between PEDF and its receptor(s) [12]. Radioligand-binding assays and crystallization analysis demonstrated that cleaved PEDF (aa24-57, aa32-380, aa78-94, and aa44–121) and N-terminal regions of PEDF are possible regions that bind to PEDF receptors [8, 24, 28].

The 44-mer peptide (aa78-121) binds to the cell surface 80-kDa protein in retinoblastoma cells and some neuronal cells, and the 44-mer peptide competes with 121I-PEDF binding in retinoblastoma Y-79 cells [24]. Notari *et al.* identified an 80-kDa phospholipase A2/nutrin/patarin-like phospholipase domain-containing 2 (PNPLA2) as a putative receptor for PEDF in retinal epithelial cells and, using a cell-free system, and showed that the interaction was involved in lipase activity of PEDF [29].

We found that a protein of molecular mass of ca. 60-kDa could be one of the candidates for PEDF receptor on endothelial cells [30]; later, 67-kDa laminin receptor (67LR) was shown to be a putative receptor

for PEDF in endothelial cells [31]. The 67LR consists of two 37-kDa laminin receptor precursor (37LRP) polypeptide chains, and both yeast two hybrid and immunoprecipitation methods revealed the interaction between PEDF and 37LPR/67LR. Further, the 25-mer peptide (aa46-70) derived from a helix-loop-helix structure of PEDF co-localized with the LR on plasma membranes and caused apoptosis of endothelial cells, inhibition of endothelial cell migration, and angiogenesis *in vitro* and *ex vivo* [31].

NUCLEAR LOCALIZATION OF PEDF

Although PEDF is a secreted protein, it is localized in the nucleus of mammalian cells [32-34]. The nuclear localization signal motif, KKRK, is located in aa146-149 domain of PEDF [35]. PEDF regulates cell cycle [36] and interacts with p53 [37-40]. Moreover, a response element specific for nuclear molecules (p63 and p73) is found in the PEDF promoter region, suggesting that PEDF is a direct target of nuclear molecules [41]. Thus, PEDF may play a crucial role in cell cycle in the nucleus.

NON-INHIBITORY SERINE PROTEASE ACTIVITY OF PEDF

PEDF is a member of the serpin superfamily. Serpins are a group of proteins with the same overall tertiary structure [42]. The C-terminal region of all serpins has an RCL that is susceptible to proteolysis [43]. The serpin active site (P1) binds to the primary specificity pocket of the target protease, leading to a change in serpin conformation from the stressed form to the relaxed form by incorporation of the serpin-exposed loop into the β-sheet. This conformational change increases stability and protects against denaturation, and the protease-serpin complex inhibits proteolytic activity [43].

Inhibitory activity against serpin proteases is not found for all serpins [44]. Although PEDF has a Leu residue at P1, which is known to be specific for inhibition of chymotrypsin and chymotrypsin-like activities, PEDF does not have typical inhibitory activities of a serpins [45]. A possible explanation for this discrepancy is that the N-terminal residues of the P1 of PEDF are unfavorable for the insertion of the serpins loop into the β-sheet of the folded serpin protein. Alternatively, alanine residues between P12 and P9 of the RCL are also known to be linked to the inhibitory property of serpins [46]. Although PEDF has an RCL like other serpins, its RCL lacks the tetrad of alanine residues between P12 and P9 [47]. In addition, the three proline residues are found in the RCL of PEDF, which could block the interaction of PEDF with target proteases [48]. Thus, changes in the RCL are likely to be responsible for non-inhibitory property of serine protease in PEDF.

ANTI-ANGIOGENIC ACTIVITY OF PEDF

PEDF exerts anti-angiogenic effects through several different mechanisms; induction of apoptosis in endothelial cells [39, 49-51], inhibition of capillary tube formation [17, 18], migration of endothelial cells [52-54], reduction of vascular endothelial growth factor (VEGF) expression [25, 55, 56] and translocation or phosphorylation of VEGF receptor 1 [57]. Although the precise underlying mechanisms for its anti-angiogenic activity are unknown, several structures in PEDF are reported to be involved in its anti-angiogenic activity. A summary of our current understanding of the structure and anti-angiogenic activity of PEDF is shown in Fig. (**1**).

Collagen type I is an angiogenic scaffold and promotes capillary tube formation through endothelial integrin engagement of collagen type 1 [58]. Collagen type 1-binding sites of PEDF, that is, the interaction of collagen type I and PEDF, play an important role in anti-angiogenic property of PEDF [17, 18, 22, 23]. In fact, mutation of the collagen type I-binding site of PEDF causes tumor progression with neovascularization in tumor xenograft study, while wild type PEDF and mutation of the heparin binding site suppresses both tumor progression and neovascularization [17].

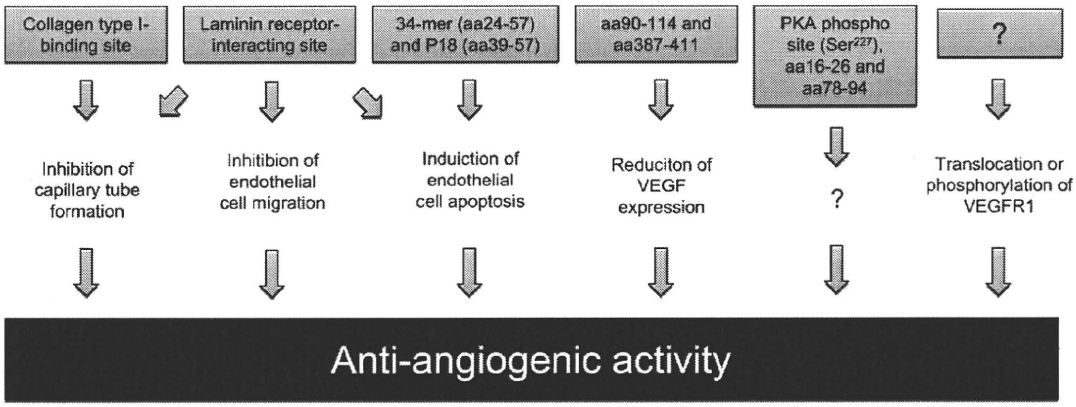

Fig. (1). Relationship between structure and anti-angiogenic activity of PEDF. VEGF, endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor-1.

A ligand-receptor interaction is also important for elicitation of divergent PEDF signals. We found that PEDF exerted anti-inflammatory properties in endothelial cells *via* the interaction with a putative PEDF receptor at a molecular mass of about 60-kDa [30]. Bernard *et al.* identified a 67LR as a PEDF receptor [31], and the 25-mer peptide (aa46-70) of PEDF bound to the LR on plasma membranes and subsequently caused anti-angiogenic reactions both *in vitro* and *ex vivo* [31].

The 34-mer peptide (aa24-57) of PEDF is also reported to act on endothelial cells and cause c-jun-NH2 kinase (JNK)-dependent endothelial cell apoptosis. This effect appears to be mediated by the inhibition of nuclear factor of activated T cells c2 (NFAT), which is regulated by JNK. A NFAT target, caspase-8 inhibitor cellular Fas-associated death domain-like interleukin 1beta-converting enzyme inhibitory protein (c-FLIP) is blocked by PEDF, which was involved in the anti-angiogenic properties of PEDF [28]. Recently, Mirochnik *et al.* analyzed the function of the 34-mer of PEDF and designed 3 peptides that covered its COOH terminus: P14 (aa43-57), P18 (aa39-57), and P23 (aa34-57) [51]. Only P18, but not P14 or P23, was found to induce apoptosis in basic fibroblast growth factor-treated or VEGF-treated endothelial cells, similar to that of the parental 34-mer peptide [51].

PEDF could exert anti-angiogenic activity by reducing VEGF levels [25, 55, 56]. Ek *et al.* generated 25-mer peptides (aa90-114 and aa387-411) and found that the peptides reduced VEGF expression in human osteosarcoma cells [55]. Although the underlying mechanisms are unclear, the protein kinase A (PKA) phosphorylation site of PEDF, Ser227 [59] and peptides aa16-26 and aa78-94 could also have anti-angiogenic activity [28].

ANTI-VASOPERMEABILITY OF PEDF

Increased vascular permeability has a pathophysiologic impact on non-proliferative diabetic retinopathy [60-63], nephritic syndrome [64-66], and hypotension [67]. Moreover, increased vascular permeability accelerates cancer invasion [68, 69]. VEGF disrupts the vascular barrier by uncoupling endothelial cell-cell junctions [70].

PEDF behaves as a functional antagonist of VEGF [71, 72]. Liu *et al.* found that the 44-mer peptide of PEDF (aa78-121) counteracted the VEGF-induced increases in vascular permeability in mouse eyes [73]. The 44-mer peptide contains the exposed elements of hC, one turn of hD, and the connecting loops [8] and a study using chimeric peptides showed that Glu101, Iso103, Leu112, and Ser115 were the amino acids responsible for the anti-vasopermeability effect of PEDF [73]. Thus, the 44-mer peptide or chimeric peptides have therapeutic potential for diseases resulting from excessive vascular permeability.

ANTI-TUMOR ACTIVITY OF PEDF

Besides its anti-angiogenic effects, PEDF also has direct anti-tumor activity by inducing tumor apoptosis and by inhibiting tumor growth and invasion [47, 74-78]. A summary of our current understanding of the structure and anti-tumor activity of PEDF is shown in Fig. (**2A** and **2B**).

Members of the family of HA-binding proteins are known to be associated with apoptosis [79-81]. PEDF contains HA-binding sites that activate caspase-8, caspase-3, and poly (ADP-ribose) polymerase, leading to apoptosis of cancer cells [2].

PEDF also acts as a tumor differentiator [9, 28, 55, 76-78, 82-87]. Alberdi *et al.* found that the 44-mer peptide (aa78-121) bound to a cell surface 80-kDa protein and induced neuronal differentiation in retinoblastoma Y-79 cells [24]. Filleur *et al.* also discovered that the 44-mer peptide (aa58-101) and its fragment (aa78-94) caused a decrease in cytokeratin K8 expression and an increase in mRNA levels of gastrin-releasing peptide/bombesin, thus suggesting the neuroendocrine differentiation of prostate adenocarcinoma cells by PEDF peptides [28]. In addition, the 25-mer peptide (aa78-102) of PEDF was also shown to suppress proliferation of osteosarcoma cells [55]. The 25-mer peptide of PEDF had similar differentiation-promoting activity in neuroblastoma [82, 86] and osteosarcoma cells [55, 83].

The extracellular matrix is deeply involved in tumor migration and invasion [88, 89]. PEDF contains an HA-binding motif and formation of PEDF-HA complexes exerts indirect anti-tumor effects by the blocking biological effects of HA, including loosening the matrix for migration and invasion [23]. Twenty five-mer peptides (aa40-64, aa78-102, and aa90-114) increased cellular adhesion to collagen type-1 [55]. Moreover, the 25-mer peptide (aa387-411) inhibits Matrigel invasion of osteosarcoma [55].

Although the precise structure has not been identified, PEDF exerts anti-tumor effects through activation of a Fas/Fas ligand pathway [25, 49] and induction of cell cycle arrest at G1 phase [87].

NEUROTROPHIC ACTIVITY OF PEDF

PEDF has neurotrophic and neuroprotective activities in the central nervous system [26, 47, 90]. The crystal structure of PEDF indicates that its neurotrophic activity is located at the exposed parts of helices C and D and at loop 90 [8]. Becerra *et al.* examined neurotrophic activity of PEDF using cleaved PEDF. Cleaved PEDF peptides (aa32-380 and aa44–121) can induce morphological differentiation and neurite outgrowth in human Y-79 retinoblastoma cells [43]. These findings suggest that the N-terminal region of PEDF is a neurotrophically active site [43]. In fact, Alberdi *et al.* generated synthetic peptides and found that the 44-mer peptide (aa78–121) derived from the N-terminal edge of PEDF had neurotrophic activity in

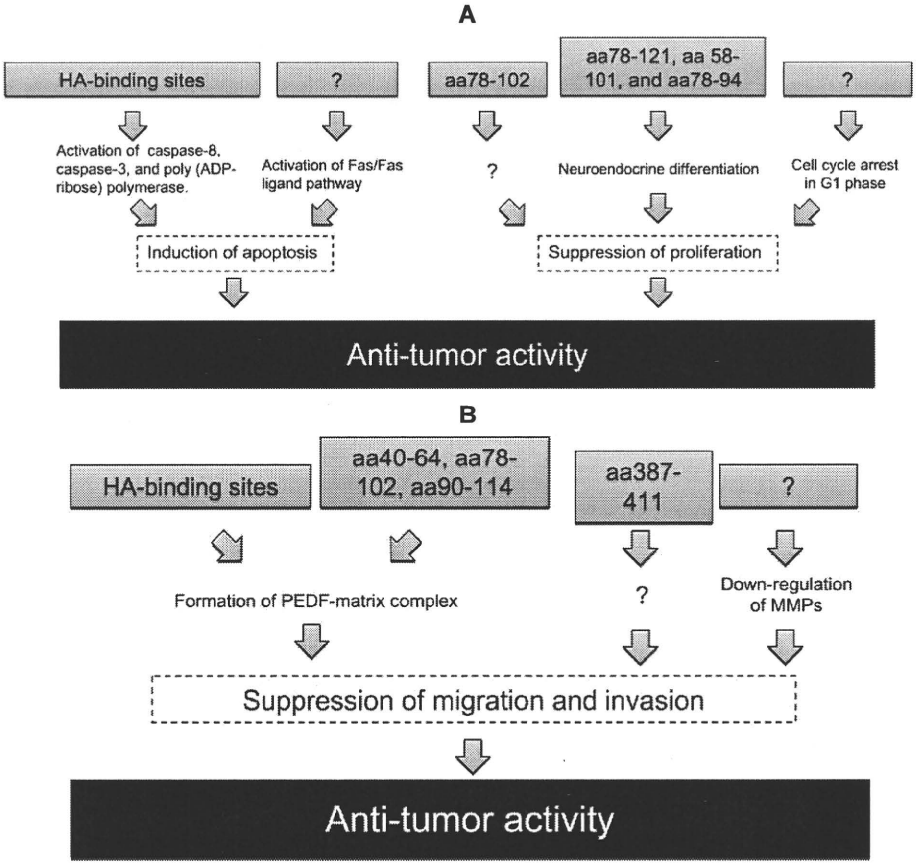

Fig. (2). Relationship between structure and anti-tumor activity of PEDF. (**A**) Apoptosis and growth suppression. (**B**) Inhibition of migration and invasion. HA, hyaluronan; MMP, matrix metalloproteinase.

retinoblastoma cells and cerebellar granuleneurons [24]. The 44-mer peptide also has a neurotrophic function in motor neurons [91] and a fragment (aa78-94) within the 44-mer can induce neuroendocrine differentiation in prostate cancer cells [28].

PEDF prevents damage to retinal ganglion cells after transient ischemia reperfusion in an ischemic rat model [92]. Li *et al.* demonstrated that the 40-mer peptide (aa82-121) protectd the retina from ischemic injury [93]. The thinning of the inner plexiform layer was also protected by the 40-mer peptide (aa82-121) of PEDF [93].

The crystal structure revealed the existence of protein kinase CK2 and PKA phosphorylation sites (Ser24 and Ser114, and Ser227) in PEDF [8]. Mutagenesis studies disclosed that protein kinase CK2 phosphorylated mutants (Ser24Glu and Ser114Glu) reduced the neurotrophic effect of PEDF, but enhanced its anti-angiogenic activity, while the PKA phosphorylation site mutant (Ser227Glu) reduced anti-angiogenic activity of PEDF [59]. These observations suggest that extracellular phosphorylation could completely change the nature of PEDF from a neutrophic to an anti-angiogenic factor.

Besides the N-terminal region of PEDF, the C-terminal site also has neuroprotective properties. A fragment from the C-terminal region (aa354-359) induces both cell differentiation and neuroprotective properties in human promyelocytic leukemia cells through inhibition of phosphatidylinositol-specific phospholipase C [94]. In addition, a fragment from the C-terminal region (aa354-359) was shown to possess neuroprotective activity and counteracts the toxic effects of beta-amyloid peptides in a rat model of Alzheimer's disease [95].

EFFECTS OF PEDF ON GLUCOSE METABOLISM

PEDF is highly expressed in the liver [96], a major organ for glucose metabolism [97-104], and PEDF has a significant role in the development of insulin resistance and the pathogenesis of diabetic complications [105-120]. Although the protein structure of PEDF that is responsible for glucose metabolism has never been determined, analysis of the genomic structure of PEDF indicates an association between PEDF and glucose metabolism.

Hepatocyte nuclear factor-4 (HNF-4), CCAAT/enhancer-binding protein homologous protein

(CHOP), and upstream stimulatory factor (USF) bind to the DNA-binding sites of the PEDF gene, which are located 200 bp upstream of the transcription start site [121]. HNF-4 enhances insulin sensitivity through activation of a phosphatidylinositol 3-kinase/Akt pathway in hepatocytes [122, 123]. CHOP induces insulin stimulation and up-regulates mammalian tribbles homologs, which is associated with insulin resistance and metabolic syndromes [124]. USF also enhances hepatic insulin signaling through up-regulation of glucokinase [125]. Moreover, HNF-4, CHOP, and USF genes are responsible for the development of diabetes mellitus [126-128]. Thus, the genomic structure of PEDF suggests an association between PEDF and glucose metabolism.

EFFECTS OF PEDF ON LIPID METABOLISM

Studies have shown the existence of high affinity PEDF-binding sites and about 80-kDa proteins in plasma membranes of various cells [24, 27, 28, 91, 129, 130]. Alberdi *et al.* found that a 44-mer peptide (aa78-121) bound to a cell surface 80-kDa protein in retinoblastoma cells and some neuronal cells [24]. Using a yeast two-hybrid screening method, Notari *et al.* found that the PEDF fragments (aa35–418, aa35–266, aa35–229, and aa35–119 of the human PEDF) bound to an 80 kDa lipase-linked membrane protein, adipose triglyceride lipase (ATGL) [29]. ATGL is a member of the newly identified calcium-independent PNPLA2 family, which possesses triglyceride lipase and acylglycerol transacylase activities [131]. In fact, PEDF is highly expressed in the liver [96], a major organ for lipid metabolism [100, 101, 132-134] and reduces hepatocyte triglyceride contents *in vitro* [135]. In a mouse model of ethanol-induced hepatic steatosis, absence of PEDF is associated with triglyceride accumulation in hepatocytes, which can be reversed by administration of exogenous PEDF [136]. Thus, PEDF modulates hepatic lipid metabolism through ATGL activation and the 44-mer peptide (aa78-121) of PEDF is a potential drug target for fatty liver diseases.

CONCLUSION

PEDF is widely expressed throughout the human body and has multiple biological activities. Administration of recombinant PEDF causes anti-tumor activity, neurotrophic activity, and ameliorates glucose and lipid metabolism, thus suggesting that PEDF is a potential therapeutic target for patients with various disorders, including cancer, neurological disorders, and the metabolic syndrome. Shorter peptides have more advantages in terms of side effects and drug delivery than full-length PEDF. PEDF consists of 418 amino acids, and it has been demonstrated that peptides derived from PEDF also possess biological activities. In this review, we summarized the known structure-function relationship of PEDF. Further study of structure-function relationship of PEDF may help us to develop peptides that can serve as new therapeutics for a broad spectrum of diseases.

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Analysis of 5′ Nontranslated Region of Hepatitis A Viral RNA Genotype I from South Korea: Comparison with Disease Severities

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Abstract

The aim of the study was to analyze genotype I hepatitis A virus (HAV) 5′ nontranslated region (NTR) sequences from a recent outbreak in South Korea and compare them with reported sequences from Japan. We collected a total of 54 acute hepatitis A patients' sera from HAV genotype I [27 severe disease (prothrombin time INR≥1.50) and 27 mild hepatitis (prothrombin time INR <1.00)], performed nested RT-PCR of 5′ NTR of HAV directly sequenced from PCR products (~300 bp), and compared them with each other. We could detect HAV 5′NTR sequences in 19 of the 54 (35.1%) cases [12 of 27 severe cases (44.4%) and 7 of 27 self-limited cases (25.9%)], all of which were subgenotype IA. Sequence analysis revealed that sequences of severe disease had 93.6%–99.0% homology and of self-limited disease 94.3%–98.6% homology, compared to subgenotype IA HAV GBM wild-type IA sequence. In this study, confirmation of the 5′NTR sequence differences between severe disease and mild disease was not carried out. Comparison with Japanese HAV A10 revealed ^{222}C to G or T substitution in 8/12 cases of severe disease and ^{222}C to G or T and ^{392}G to A substitutions in 5/7 and 4/7 cases of mild disease, respectively, although the nucleotide sequences in this study showed high homology (93.6%–100%). In conclusion, HAV 5′NTR subgenotype IA from Korea had relatively high homology to Japanese sequences previously reported from Japan, and this region would be considered one of the antiviral targets. Further studies will be needed.

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Introduction

Although hepatitis A vaccination is highly effective, providing herd protection and decreasing mortality and morbidity related to the hepatitis A virus (HAV) [1–3], HAV is still a common cause of hepatitis reportedly leading to occasional lethal acute liver failure in many countries of the world [4–7]. Recently, a rise in the frequency of hepatitis A outbreaks was observed in South Korea, which lies adjacent to Japan, while the number of adult hepatitis A cases in Japan has been progressively decreasing during the last several years. There is a concern regarding a possible HAV epidemic in Japan in the near future, as universal vaccination against hepatitis A is not performed in this country.

HAV is a member of the genus *Hepatovirus* in the *Picornaviridae* family, and has a positive-sense single-stranded RNA genome approximately 7.5 kb in length [8]. The genome codes a large open reading frame (ORF), which is flanked by 5′ nontranslated region (5′NTR) and 3′NTR. The downstream part of 5′NTR represents the internal ribosomal entry site (IRES), which mediates cap-independent translation initiation and is important for HAV replication [9,10]. 5′NTR of HAV is also known as one of the most highly conserved in the HAV genome sequences, making this region one of the likely candidates for antiviral targets [9,11]. It was reported that nucleotide variations in the central portion of 5′NTR of HAV may influence the severity of hepatitis A [12].

Human HAV strains can be grouped into four genotypes (I, II, III and IV) and unique simian strains belong to three additional genotypes (IV, V and VI). Between each of these genotypes, the nucleotide sequence varies by 15–20% of the base positions in the P1 region [13]. Genotype I is the most abundant type worldwide, and genotype IA in particular has been reported from North America, Korea, China, Japan and Thailand [14].

The aim of this study is to characterize the recent HAV genotype I 5′NTR sequences in Korea, to compare them with those reported from Japan and to clarify this region as a target candidate for anti-HAV drugs.

Materials and Methods

Patients

Fifty-four patients infected with HAV subgenotypes IA and IB were included in this study. Serum samples were collected at four

hospitals located in the Seongnam city area, near Seoul, South Korea. Our study was approved by the Seoul National University Bundang Hospital Institutional Review Board (IRB), and we obtained written informed consent from every patient enrolled during Sep 2008 to Aug 2008. We collected serum or plasma samples immediately after hospital admission, and they were stored at −70°C. The 54 patients comprised 27 with severe disease, defined as prolonged prothrombin time [international normalized ratio (INR) > or = 1.5] and 27 with mild disease: self-limited acute hepatitis in this study (Table S1A & S1B).

Primers for PCR and Direct Sequencing

For amplification of HAV sequences and bidirectional direct sequencing of the amplified segments, we prepared several primers for PCR and sequencing as previously described [12]. These primers were prepared with the sequence reported by Cohen et al [8].

Detection of Hepatitis A Virus RNA in Serum

RNA was extracted from sera using the acid guanidinium-phenol-chloroform method. Reverse transcription was performed with HAV genome specific antisense primer (5′-AGTACCTCA-GAGGCAAACAC-3′) as previously described [12].

In the first round PCR, 1 μl of 20 μl of the cDNA solution was used. The first round PCR was performed with 50 μl of reaction mixture containing 25 pmol of outer antisense primer (5′-AGTACCTCAGAGGCAAACAC-3′) and sense primer (5′-TCTTGGAAGTCCATGGTGAG-3′), 200 μM of each dNTP, 50 mM KCl, 10 mM Tris HCL (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, and 2.5 units of Ex Taq polymerase (Takara Bio Inc., Ohtsu, Shiga, Japan). Amplification conditions consisted of 35 cycles of 95°C for one minute, 50°C for one minute, and 72°C for one minute, and 1 μl of the first round product was used for the second round of PCR with the same PCR mixture, except 1.0 μM of inner sense primer (5′-GGGACTTGATACCT-CACCGC-3′) and antisense primer (5′-CCACATAAGGCCC-CAAAGAA-3′) were used. Amplification conditions for the second round were the same as those for the first round. The second-round PCR products (6 μl) were analyzed by 8% polyacrylamide gel electrophoresis, stained with SyBr green (Takara), and visualized by UV transillumination. In all experiments, the negative samples showed negative results for HAV RNA. HAV genotypes were determined by previously described methods based on the VP1-P2A region [14].

Direct Sequencing of HAV cDNA Fragments

To prepare the sequence template (nucleotides 75-638 of 5′NTR of HAV), PCR products were treated with ExoSAP-ITR (Affymetrix, Inc., Santa Clara, CA), and then sequenced using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were analyzed using Applied Biosystems 3730xl (Life Technologies).

Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported in this article will appear in GenBank nucleotide sequence databases with accession numbers AB571027 to AB571045.

Phylogenetic Analysis

To examine the heterogeneity of the viral sequences obtained, a phylogenetic tree was constructed using the neighbor joining methods. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 10,000 times. These analyses were conducted with the Genetyx-WIN program, version 10 (Software Development, Tokyo, Japan).

Statistical analysis

Differences in proportions among the groups were compared by Fisher's exact probability test, Student's t test and Welch's t test.

Results

Clinical Features of Patients with Acute Hepatitis A Genotype 1 in Korea

Characteristics of these patients at admission are summarized in Table S1. There were no differences in age and gender ratio between the severe and mild disease groups. Mean age of the severe and mild disease groups was 32.1±6.1 and 32.6±5.8 years, respectively. Male gender was dominant in both groups (male/female: 19/8 and 18/9 in the severe and mild disease groups, respectively). Almost all patients of both groups were subgenotype IA, with only two and one being subgenotype IB in the severe and mild disease groups, respectively.

Sequence Analysis of Korean Isolates

Although the VP1/2A region could be detected in the same serum or stool samples of the same patients, we could detect HAV 5′NTR sequences in 19 of the 54 (35.1%) cases [12 of 27 severe cases (44.4%) and 7 of 27 self-limited cases (25.9%)] by reverse-transcription-nested PCR. All these sequences were subgenotype IA. Then we performed further sequence analysis in these 19 patients by the methods of Fujiwara et al [12]. Japanese studies showed that fewer nucleotide variations were found between nucleotides 200 and 500 of 5′NTR in cases of fulminant hepatitis and severe acute hepatitis than in cases of self-limited acute hepatitis [12]. We thusly performed sequence analysis of the region between nucleotides 200 and 500.

Sequences between nucleotides 200 and 500 were then compared with the wild-type HAV GBM/WT RNA (X75215) [15]. The nucleotide sequence identities of 5′NTR from severe and mild cases ranged from 93.6% to 99.0% and from 94.3% to 98.6%, respectively, compared with wild-type HAV GBM sequence. The distribution of nucleotide variations is shown in Table S2A & S2B. Sequences from cases of severe and mild diseases were mostly similar. Although there was no statistical significance, ^{214}C, ^{220}T and ^{464}T were found in one case each of the mild disease group (Table S2B). On the other hand, ^{227}deletion of nucleotide and ^{382}A, respectively, were found in two and one cases of the severe disease group (Table S2A). The number of nucleotide substitutions is shown in Figure 1A & 1B. The average number of substitutions between nucleotides 200 and 500 was 10.8 (6.8) [mean (SD)] per case in severe disease and 6.8 (4.5) in mild disease. Differences between severe and mild cases were not statistically significant. We could not construct a phylogenetic tree using these sequences (data not shown).

Comparison to Japanese HAV Sequences Reported from 1984 to 1999

5′NTR of HAV possesses a secondary structure including stems and loops, functions as an IRES, and plays an important role in translation and replication of this virus [9,16]. There are six domains in IRES, which is located between nucleotides 151 and 734. Portions of domains III and IV are present between nucleotides 200 and 500. Domain III is located between nucleotides 99 and 323, and domain IV is located between nucleotides 324 and 586. The region between nucleotides 203 and

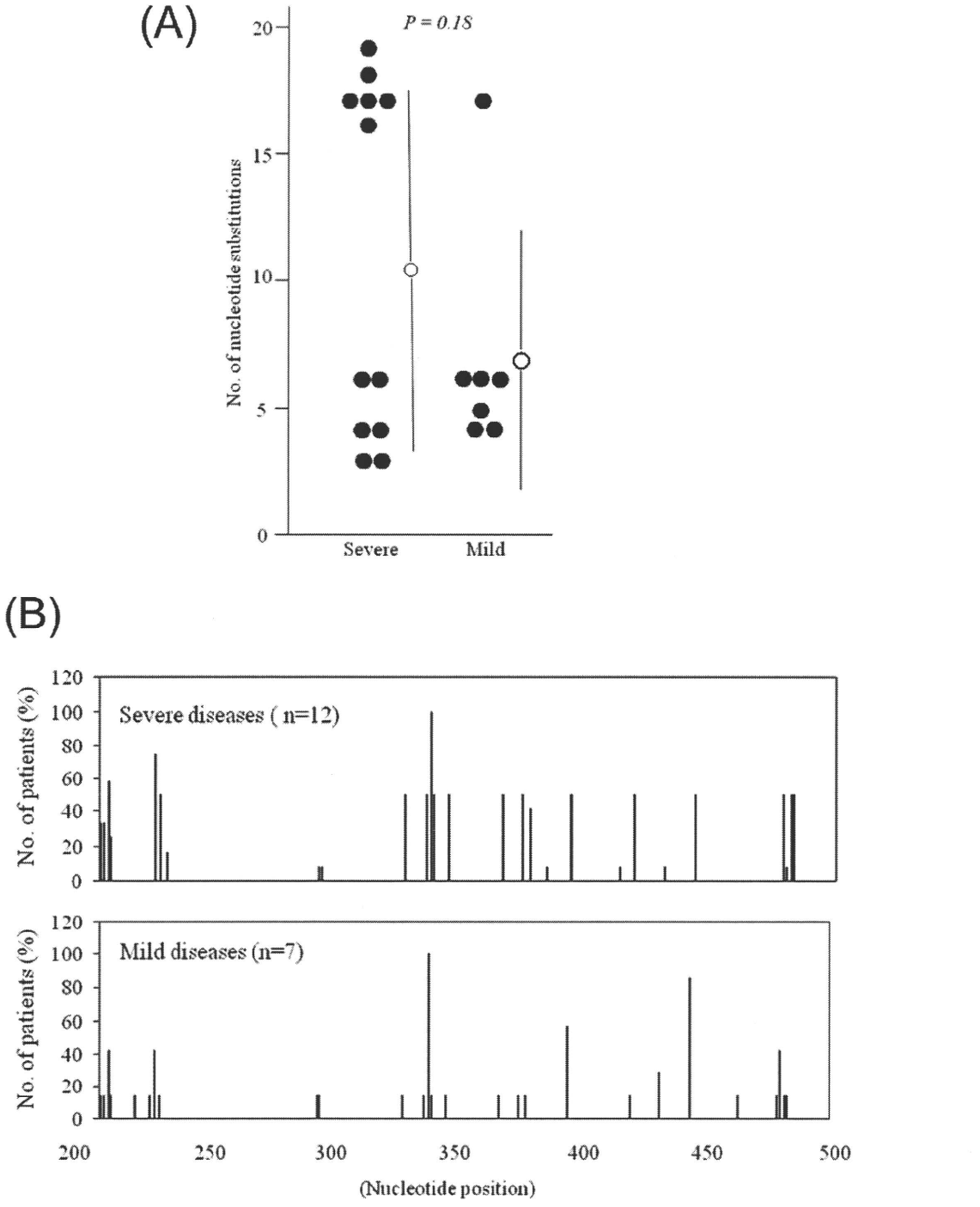

Figure 1. Disease severity and nucleotide substitutions in HAV IRES when compared with HAV GBM. (**A**) Number of nucleotide substitutions between nucleotides 200 and 500. Nucleotide sequences were compared with HAV GBM/WT RNA (X75215) [15]. Bars represent mean (SD). Severe, severe disease; Mild, mild disease. (**B**) Distribution of nucleotide substitutions between nucleotides 200 and 500 of the 5′ non-translated region. Bars indicate the percentage of cases with substitutions at each nucleotide position.
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250 is particularly pyrimidine-rich. To examine the homology with the HAV sequences from Japan reported by Fujiwara et al. [12], we compared the sequences from nucleotides 200 to 500 with A10 (AB045328) from Japan [12]. The nucleotide sequence identities of 5′NTR from severe and mild disease groups ranged from 94.3% to 99.6% and from 93.6% to 100%, respectively, compared with the HAV A10 sequence [12] (Table S3A & S3B). In the Korean group, we found ^{222}C to G or T substitution in 8/12 cases of severe disease and ^{222}C to G or T and ^{392}G to A substitutions in 5/7 and 4/7 cases of mild disease, respectively.

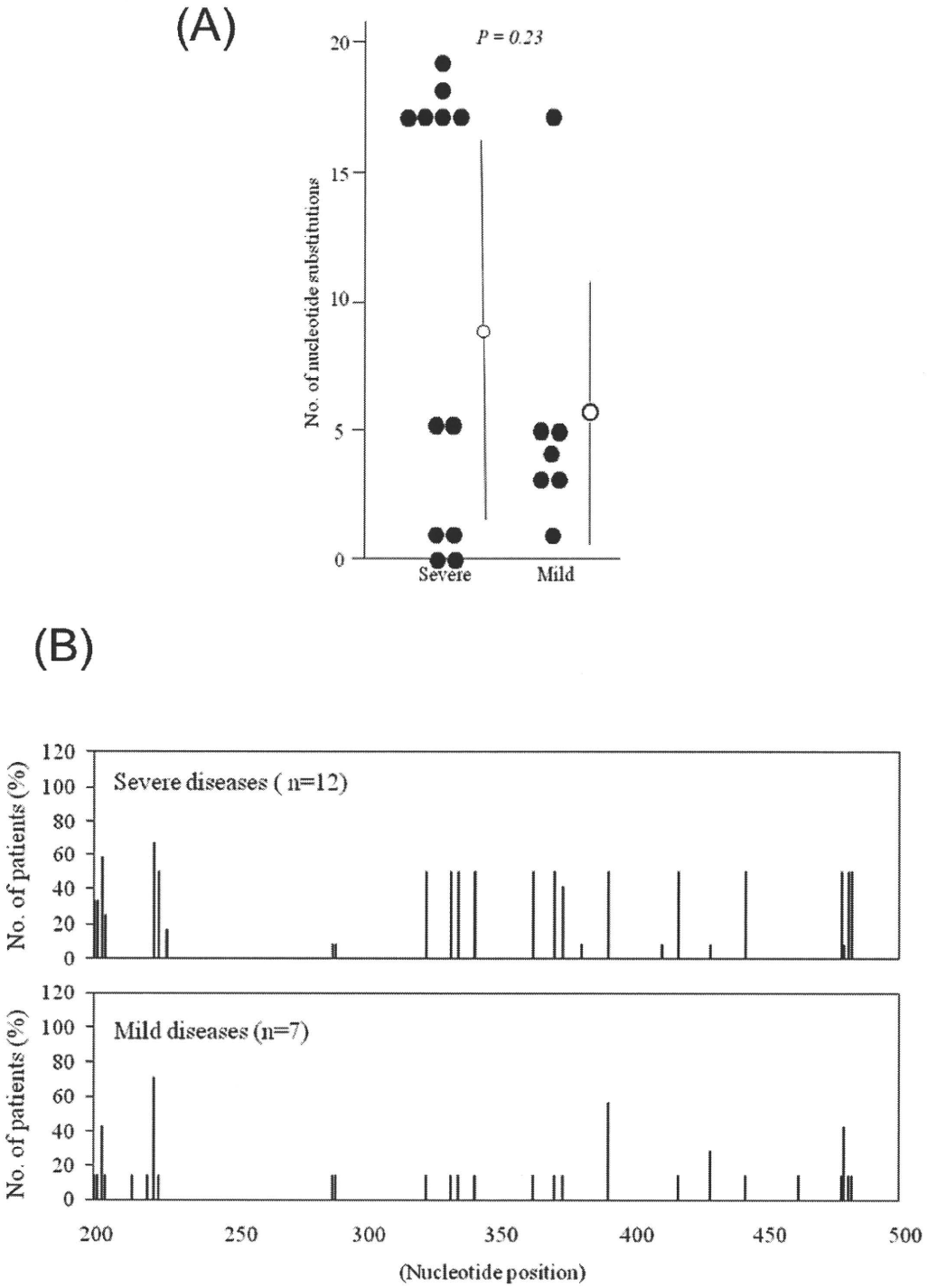

Figure 2. Disease severity and nucleotide substitutions in HAV IRES when compared with HAV A10. (**A**) Number of nucleotide substitutions between nucleotides 200 and 500 Nucleotide sequences were compared with A10 (AB045328) from Japan [12]. Bars represent mean (SD). Severe, severe disease; Mild, mild disease. (**B**) Distribution of nucleotide substitutions between nucleotides 200 and 500 of the 5′ non-translated region. Bars indicate the percentage of cases with substitutions at each nucleotide position.
doi:10.1371/journal.pone.0015139.g002

The number of nucleotide substitutions is shown in Figure 2A & 2B, with the average number between nucleotides 200 and 500 being 9.7 (8.2) [mean (SD)] per case in severe disease and 5.4 (5.2) in mild disease. Again, differences between severe and mild cases were not statistically significant.

Discussion

The number of adult hepatitis A cases has been progressively increasing during the last several years in Korea [6,14]. In Japan, on the other hand, the number of patients with sporadic type A

hepatitis has recently been on the decrease. In the 9 years from 1999 inclusive, 763, 381, 491, 502, 303, 139, 170, 320 and 157 hepatitis A cases were reported to the Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan (www.nih.go.jp). Japan lies adjacent to Korea, separated by the Sea of Japan. The two countries have some cultural similarities. In Japan, there is no universal vaccination program against hepatitis A and hepatitis B. These circumstances have raised concerns about a possible HAV epidemic in Japan. We then analyzed HAV genome sequences from Korea and compared them with the reported sequences from Japan over the past several years.

In the present study, as most of the HAV strains belonged to subgenotype IA in Korea [14], we chose only genotype I patients for analysis. Among 54 HAV IgM positive sera, 35.1% (n = 19) were positive for HAV RNA by nested RT-PCR for 5′NTR. All these strains belonged to subgenotype IA. We tried to perform phylogenetic tree analysis, but these 19 strains formed a single cluster to which almost all Japanese sequences reported by Fujiwara et al [12] belonged (data not shown). Fujiwara et al [12] found an association between the severity of hepatitis A and nucleotide variations in 5′NTR of Japanese HAV RNA. In the present study, we did not confirm 5′NTR sequence differences between severe disease and mild disease.

The age of HAV sequence-analyzed patients in the present study was 30.5±5.9 and 31.4±5.0 years, respectively, in severe and mild diseases. The gender of HAV sequence-analyzed patients was male-dominant (male/female: 8/4 and 6/1 in the severe disease and mild disease groups, respectively). In the study by Fujiwara et al [12], the patients were also male-dominant, but their age with fulminant hepatitis and severe acute hepatitis (43.1±14.4 year, $P = 0.010$ and 41.6±12.6, $P = 0.010$, respectively) was significantly higher than the age of severe-disease patients. On the other hand, the age of their patients with self-limited acute hepatitis was similar to that of our mild-disease patients. We defined patients with prothrombin time INR ≥1.50 as severe hepatitis in this study, whereas Fujiwara et al [12] defined patients with prothrombin time of less than 40% as severe hepatitis with (fulminant hepatitis) or without encephalopathy (severe acute hepatitis).

In Japan, similar to the situation in Korea [6], young adults seem not to have protective antibody against HAV, and so it appears that hepatitis A cases can be expected to increase in the near future.

A previous study showed that the 5′ border of IRES is located between nucleotides 151 and 257, while the 3′ border extends to the 3′ end of 5′NTR, between nucleotide 695 and the first initiation codon at 735 [17]. ^{222}C to G or T substitution was located on the loop structure at domain IIIa of HAV IRES. A previous Japanese study showed that nucleotide 225 substitutions occurred in 80% of the sequences around nucleotide position 222 [12]. ^{392}G to A substitution located at domain IV of HAV IRES was observed in 64.2% (9/14) of the Korean HAV sequences. Fujiwara et al [12] also reported that substitutions at nucleotide 391 were seen in 32% of Japanese HAV patients. It is possible that these substitutions were non-specific mutations.

In conclusion, HAV 5′NTR subgenotype IA from Korea had relatively high homology to the Japanese sequences previously reported, and this region may represent a viable antiviral target. In Japan, as in Korea, the introduction of childhood vaccination and catch-up vaccination for adolescents and young adults should be considered.

Supporting Information

Table S1 Patient Characteristics. (A) Severe disease, (B) Mild disease.
(DOC)

Table S2 Comparison of the nucleotide sequences of the HAV 5′ non-translated region with GBM. (A) Severe disease, (B) Mild disease. The consensus sequence for HAV GBM/WT RNA (X75215) [15] is shown on the top. Dots indicate conserved nucleotides; differences are shown by the appropriate single letter nucleotide. -, deletion mutant.
(DOC)

Table S3 Comparison of the nucleotide sequences of the HAV 5′ non-translated region with GBM. (A) Severe disease, (B) Mild disease. The consensus sequence for A10 (AB045328) from Japan [12] is shown on the top. Dots indicate conserved nucleotides; differences are shown by the appropriate single letter nucleotide. -, deletion mutant.
(DOC)

Acknowledgments

We dedicate this work in the memory of the late Dr. Koji Yano, Clinical Research Center, NHO Nagasaki Medical Center, Omura, Nagasaki, Japan.

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Author Contributions

Conceived and designed the experiments: TK SHJ FI OY. Performed the experiments: TK SHJ. Analyzed the data: TK SHJ KF. Contributed reagents/materials/analysis tools: TK SHJ FI KF OY. Wrote the paper: TK SHJ KF. Collected the samples: SHJ.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The requirement for a sufficient period of corticosteroid treatment in combination with nucleoside analogue for severe acute exacerbation of chronic hepatitis B

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Abstract
Background The prognosis of severe acute exacerbation of chronic hepatitis B is very poor if signs of liver failure appear. We have reported the efficacy of the early introduction of sufficient doses of corticosteroids (CSs) and nucleoside analogues (NAs), but the optimal period of immunosuppressive therapy has not been well demonstrated. In this study, we analyzed patients with severe acute exacerbation of chronic hepatitis B treated with CSs and NAs, prospectively, in order to clarify the factors affecting their outcome.
Methods Ten patients, admitted to our liver unit between 2000 and 2009, were defined as having severe exacerbation of chronic hepatitis B based on our uniform criteria, and were enrolled in this study. NAs and sufficient doses of CS were introduced as soon as possible after making the diagnosis of severe disease prospectively.
Results Seven of the 10 patients recovered. The absence of fulminant hepatitis on admission, the improvement of prothrombin time (PT) activity and the decline of hepatitis B virus (HBV) DNA during the first 2 and 4 weeks, respectively, were significant in the recovered patients, while the worsening of total bilirubin level during 4 weeks, especially between week 2 and week 4, was significant in those who died.
Conclusions In severe acute exacerbation of chronic hepatitis B, more than a few weeks of CS treatment in combination with an NA is required in the early stage, whereas a short period of conventional pulse therapy would be insufficient for treating this condition.

Keywords Chronic hepatitis B · Severe exacerbation · Corticosteroid · Nucleoside analogue

Abbreviations
HBV Hepatitis B virus
CS Corticosteroid
NA Nucleoside analogue

Introduction

Exacerbation of hepatitis B in chronic hepatitis B virus (HBV) carriers may occur spontaneously or in relation to cytotoxic or immunomodulatory therapy. A clinical picture ranging from anicteric hepatitis to severe exacerbation, sometimes fulminant liver failure, may develop, and it is associated with high mortality [1]. In a retrospective Japanese survey of HBV carriers with hematologic malignancies, a 53% incidence of severe hepatitis with a 24% mortality rate was reported in relation to chemotherapy [2]. For the treatment of patients with severe exacerbation, liver transplantation may be a viable option, although it is contraindicated in patients with underlying malignancies. However, the problem of the shortage of donor livers still remains in Japan. Thus, therapies other than transplantation must be further investigated.

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In HBV infection, liver injury is considered to be induced mainly by cytotoxic T-lymphocyte-mediated cytolytic pathways in HBV-infected hepatocytes [3], and it was suggested that treating chronic hepatitis B patients with corticosteroids (CSs) in order to inhibit an excessive immune response and prevent cytolysis of infected hepatocytes would be reasonable, if the HBV could be controlled [4]. However, the advantage of CSs in the treatment of chronic active hepatitis B was not confirmed by control studies, and their use for routine management has fallen out of favor [5–7], although those studies mainly dealt with cases of clinically "nonsevere" hepatitis.

As to the effects of CS treatment for "severe and potentially life-threatening" exacerbation of chronic hepatitis B, Lau et al. [8] reported that the reintroduction of long-term high-dose CS in the early phase of reactivation after the withdrawal of immunosuppressive therapy prevented both progressive clinical deterioration and the potential need for orthotopic liver transplantation.

Recently, nucleoside analogues (NAs) have been administered safely even in severe disease [9–13], and in our previous studies, we reported that the introduction of high-dose CS and NA could significantly reverse deterioration in patients with "clinically severe, life-threatening" exacerbation of chronic hepatitis B compared with historical controls, when used in the early stage of the illness [14, 15]. But the dose and the period of CS use have still to be clarified.

In this study, we analyzed patients with clinically severe exacerbation of chronic hepatitis B treated with the initiation of sufficient doses of CS and NA prospectively, in order to clarify the factors affecting the outcome and the optimal period of sufficient CS therapy required to suppress an excessive host immune response.

Patients and methods

Patients

Ten patients with severe acute exacerbation of chronic hepatitis B admitted to our liver unit (Chiba University Hospital and related hospitals) between 2000 and 2009 were studied. The diagnosis of a chronic hepatitis B viral carrier state was made based on either the positivity of hepatitis B surface antigen (HBsAg) for at least 6 months before entry or, in patients with follow-up periods less than 6 months before entry, it was based on the positivity of HBsAg, presence of anti-hepatitis B core antibody (HBcAb) at a high titer, and negativity or a low titer of IgM anti-hepatitis B core antibody (IgM-HBc). Patients fulfilling all the following three criteria during the course were defined as having severe exacerbation: prothrombin time (PT) activity less than 60% of normal control, total bilirubin (T-Bil) greater than 3.0 mg/dl, and alanine transaminase (ALT) greater than 300 IU/l during the course. Patients with PT activity less than 40% of control and hepatic encephalopathy were defined as having fulminant hepatitis. All patients were in poor general condition, including general malaise, fatigue, jaundice, edema, ascites, and encephalopathy. Histological examination was performed in the convalescent phase or after the death of patients. The work described in this manuscript has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and the guidelines of the ethics committee of our institutional review boards. Informed consent was obtained from all patients or appropriate family members.

All patients were negative for IgM anti-HAV antibody, anti-hepatitis D antibody, anti-HCV antibody, HCV RNA, IgM anti-Epstein–Barr virus antibody (IgM-EBV), IgM anti-herpes simplex virus antibody (IgM-HSV), IgM anti-cytomegalovirus antibody (IgM-CMV), anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody-1, and anti-mitochondrial antibody. Patients with histories of recent exposure to drugs and chemical agents as well as those with recent heavy alcohol intake were ruled out. One patient was HIV-positive but had no clinical evidence of acquired immune deficiency syndrome.

Treatment protocols

All patients treated were examined prospectively. Informed consent was obtained from the patients or appropriate family members. Patients were treated with the NA, lamivudine (LMV) before 2007 or with entecavir (ETV) after 2007, and CS. Early introduction of CS was defined as follows: 40 mg or more of prednisolone (PSL) daily was administered within 10 days after the diagnosis of severe disease, using the above-mentioned criteria. This dosage was maintained for a minimum of 4 days. When the patient showed a trend toward remission of PT, the dosage was reduced by 10 mg at least every 4 days and tapered off. Patients for whom more than 10 days had already passed after the diagnosis before they had been admitted to our unit were treated with delayed introduction of CS (delayed CS). Patients with marked prolongation of PT were treated with 1000 mg of methylprednisolone (MPSL) daily for 3 days followed by the same PSL therapy as that described above.

LMV was administered at a daily dose of 100–300 mg. ETV was administered at a daily dose of 0.5–1.0 mg. Patients were also treated with intravenous glycyrrhizin (stronger neominophagen C; SNMC), an aqueous extract of licorice root, at 60–100 ml/day this agent is reported to

have anti-inflammatory activity and has been used for the treatment of chronic viral hepatitis in Japan [16].

Serological markers

HBsAg, hepatitis B envelope antigen (HBeAg), anti-HBe antibody (HBeAb), HBcAb, IgM-HBc, IgM anti-HAV antibody, and anti-hepatitis D antibody were detected by commercial radioimmunoassay (Abbott Laboratories, Chicago, IL, USA), and HCV RNA was measured by nested reverse transcription polymerase chain reaction (RT-PCR) [17]. Second-generation anti-HCV antibody was measured by enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan). IgM-EBV, IgM-CMV, and IgM-HSV were examined by enzyme-linked immunosorbent assays. Antinuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, and anti-liver kidney microsomal-1 antibody were examined by a fluorescent antibody method. HBV DNA level was measured by Amplicor HBV monitor (Roche Diagnostics, Tokyo, Japan).

Statistical analysis

Differences in proportions among groups were compared by Fisher's exact probability test, Student's *t*-test, and Welch's *t*-test.

Results

Clinical features of severe chronic hepatitis B patients on admission

Of the 10 patients, 8 were men and 2 women. Mean age at the time of admission was 49.2 ± 11.2 years. Four patients had primary diseases and conditions (one non-Hodgkin's lymphoma, one rheumatoid arthritis after a curative operation for hepatoma, one HIV-positive without immunodeficiency, and one gastrointestinal stromal tumor), and 3 had been treated with immunosuppressive or cytotoxic drugs, suffering exacerbations after their withdrawal. Four patients were diagnosed with fulminant hepatitis on admission (Table 1).

Mean PT activity was 32 ± 10%, mean ALT 894 ± 596 IU/l, and mean T-Bil 12.4 ± 8.5 mg/dl. HBeAg/HBeAb status was +/− in 3, −/+ in 6, and +/+ in 1. Mean HBV DNA was 6.2 ± 1.6 logcopy/ml, and precore/core promoter mutation status is shown in Table 2. Mean alfa-fetoprotein (AFP) was 250.8 ± 293.1 ng/ml and mean hepatocyte growth factor (HGF) was 7.0 ± 10.6 ng/ml (Table 2).

Types of therapies

As the initial CS, 1000 mg of MPSL was introduced to 4 patients, 60 mg of PSL to 5, and 40 mg of PSL to one. The mean duration between the diagnosis of severe disease and introduction of CS was 6.4 ± 4.8 days, and the mean duration of CS therapy was 63.6 ± 56.5 days. Eight patients were treated with early CS and 2 with delayed CS. As the NA, LMV was introduced to 7 patients and ETV to 3. In the 4 patients with fulminant hepatitis, artificial liver support (plasma exchange and hemodiafiltration) was performed (Table 3).

Biochemical responses to therapy

Changes in PT activities, ALT levels, T-Bil levels, and HBV DNA levels after the introduction of combination therapy are shown in Fig. 1. Mean PT activity was 34 ± 10% before initiation of the combination therapy (week 0), 58 ± 23 at 2 weeks after starting (week 2), and 62 ± 30 at 4 weeks (week 4). The improvement in PT activity was significant between week 0 and 2, and between week 0 and 4 ($p = 0.01$ and $p = 0.02$, respectively)

Table 1 Clinical features of patients

Patient	Age (years)	Sex	Onset	Complication	History of immunosuppressive or cytotoxic therapy	Fulminant hepatitis on admission
1	43	M	2000		−	−
2	30	M	2002		−	−
3	35	M	2002		−	−
4	45	M	2003	HIV (+)	−	+
5	55	M	2004		−	+
6	63	F	2005		−	+
7	65	M	2006	Rheumatoid arthritis, post-operation for hepatoma	+	+
8	55	F	2007	Non-Hodgkin's lymphoma	+	−
9	51	M	2007		−	−
10	50	M	2009	Gastrointestinal stromal tumor	+	−

Table 2 Biochemical, virological and histological features of patients

Patient	PT (%)	ALT (IU/l)	T-Bil (mg/dl)	D-Bil (mg/dl)	α-Fetoprotein (ng/ml)	Hepatocyte growth factor (ng/ml)	Liver histology	HBeAg	HBeAb	HBV DNA (logcopy/ml)	Precore mutation	Core promoter mutation
1	28	1463	9.1	5.8	658.9	2.07	CH (F3, severe)	+	−	8.6	Wild	Mutant
2	26	1897	19.0	15.1	106.5	2.04	CH (F2, severe)	+	−	7.2	ND	ND
3	24	723	9.3	7.4	18.9	0.75	CH (F3, severe)	−	+	7.2	Mixed	Wild
4	32	278	10.6	4.5	672.8	33.69	ND	−	+	5.2	Mutant	Wild
5	32	129	12.7	7.6	103.0	3.68	ND	−	+	4.2	Mixed	Mutant
6	30	968	9.9	4.9	674.2	9.80	Massive necrosis	+	+	4.3	Mixed	Mutant
7	21	364	10.5	6.6	184.4	9.37	ND	−	+	5.8	Mixed	Mutant
8	57	1595	3.0	2.0	6.8	ND	ND	−	+	7.6	ND	ND
9	35	938	6.6	4.8	20.8	0.39	ND	+	−	7.5	Wild	Mutant
10	34	584	33.5	26.0	61.6	1.15	CH (F3, severe)	−	+	4.8	Mixed	Mutant

PT prothrombin time, *ALT* alanine transaminase, *T-Bil* total bilirubin, *D-Bil* direct bilirubin, *HBeAg* hepatitis B envelope antigen, *HBeAb* hepatitis B envelope antibody, *HBV* hepatitis B virus, *ND* not done, *CH* chronic hepatitis

(Fig. 1a). The mean ALT level was 1260 ± 539 IU/l at week 0, 101 ± 77 at week 2, and 50 ± 33 at week 4. The ALT levels fell in all patients during the treatment course, with the decline between weeks 0 and 2, and between weeks 0 and 4 being significant ($p < 0.001$) (Fig. 1b). The mean T-Bil level was 11.9 ± 9.1 mg/dl at week 0, 10.2 ± 8.2 at week 2, and 9.9 ± 9.5 at week 4. Changes in T-Bil levels were not significant in 4 weeks (Fig. 1c). HBV DNA was 6.4 ± 1.6 log copies/ml at week 0, 4.7 ± 1.4 at week 2, and 3.9 ± 1.4 at week 4. The differences were significant between weeks 0 and 2 and weeks 0 and 4 ($p = 0.02$ and $p = 0.001$, respectively) (Fig. 1d).

Comparison of backgrounds on admission between recovered patients and nonsurvivors

Differences in age, sex, a history of immunosuppressive or cytotoxic therapy, PT activity, ALT level, T-Bil level, HBV DNA level, HBeAg/HBeAb status, AFP level, and HGF level on admission were not significant between recovered patients and nonsurvivors. The presence of fulminant hepatitis on admission was significantly different between the two patient groups ($p = 0.03$).

Comparison of responses to therapy between recovered patients and nonsurvivors

The mean PT activity was 32 ± 5% at week 0, 63 ± 21 at week 2, and 67 ± 23 at week 4 in the recovered patients, and 30 ± 7%, 36 ± 4, and 27 ± 6 in the nonsurvivors. Improvement of PT activity was significant between weeks 0 and 2, and between weeks 0 and 4 ($p = 0.003$ and $p = 0.006$, respectively) in the recovered patients, but there was no significant difference in PT activity levels in the non-survivors.

The mean ALT level was 1068 ± 589 IU/l at week 0, 81 ± 31 at week 2, and 59 ± 39 at week 4 in the recovered patients, and 1387 ± 57 IU/l, 80 ± 78, and 30 ± 5 in the nonsurvivors. The ALT levels fell significantly between weeks 0 and 2, and between weeks 0 and 4 in both groups ($p < 0.005$).

The mean T-Bil level was 15.8 ± 9.8 mg/dl at week 0, 10.3 ± 10.0 at week 2, and 7.7 ± 8.4 at week 4 in the recovered patients, and 7.2 ± 3.8 mg/dl, 13.1 ± 2.2, and 21.1 ± 2.5 in the nonsurvivors. Changes in T-Bil levels were not significant during 4 weeks in the recovered patients, but the increases between weeks 0 and 4, and between weeks 2 and 4 were significant in the nonsurvivors ($p = 0.006$ and $p = 0.02$, respectively).

The mean HBV DNA was 6.7 ± 1.6 log copies/ml at week 0, 4.7 ± 1.7 at week 2, and 3.7 ± 1.5 at week 4 in the recovered patients, and 5.7 ± 1.8 log copies/ml, 4.8 ± 0.9, and 4.3 ± 1.5 in the nonsurvivors. HBV DNA

Table 3 Therapies and responses

Patient	Duration between diagnosis of severe disease and introduction of corticosteroid (days)	Corticosteroid therapy		Nucleoside analogue	Artificial liver support	Outcome
		Drug	Period (days)			
1	4	MPSL + PSL	63	Lamivudine	–	Recovery
2	3	PSL	183	Lamivudine	–	Recovery
3	3	PSL	150	Lamivudine	–	Recovery
4	3	MPSL + PSL	21	Lamivudine	PE + CHDF	Death
5	8	PSL	32	Lamivudine	PE + CHDF	Recovery
6	9	PSL	37	Lamivudine	PE + CHDF	Death
7	12	PSL	35	Lamivudine	PE + CHDF	Death
8	1	PSL	60	Entecavir	–	Recovery
9	5	MPSL + PSL	31	Entecavir	–	Recovery
10	16	MPSL + PSL	24	Entecavir	–	Recovery

PSL prednisolone, *MPSL* methylprednisolone, *PE* plasma exchange, *CHDF* continuous hemodiafiltration

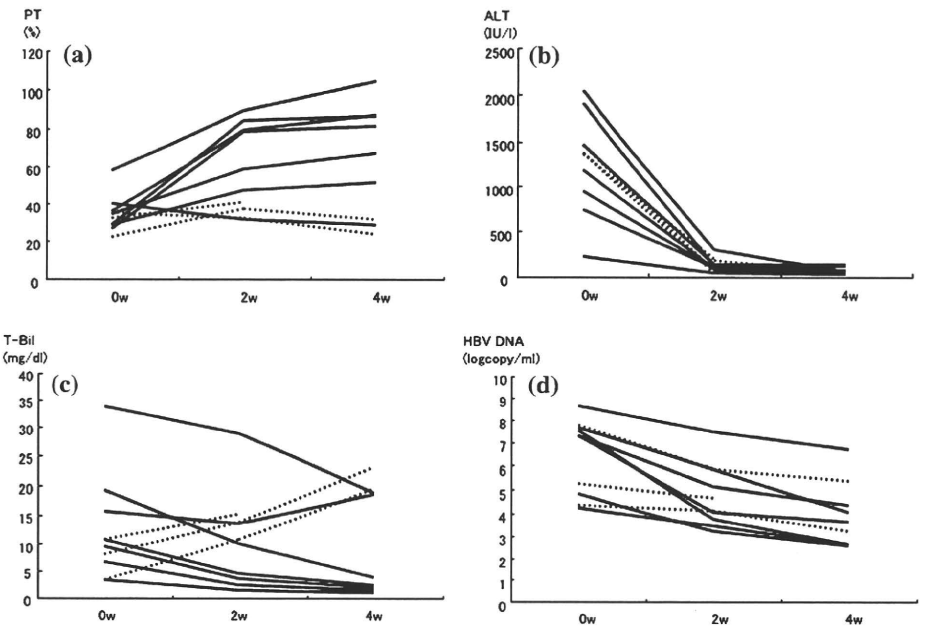

Fig. 1 Prothrombin time (*PT*) activities (**a**), alanine transaminase (*ALT*) levels (**b**), total bilirubin (*T-Bil*) levels (**c**), and hepatitis B virus (*HBV*) DNA levels (**d**) before and after treatment in 7 recovered patients and 3 nonsurvivors. *Solid and dashed lines* denote values for recovered and dead patients, respectively. *w*, Weeks

declined significantly between weeks 0 and 2, and between weeks 0 and 4 in the recovered patients ($p = 0.04$ and $p = 0.003$, respectively). In contrast, the decline of the HBV load was not significant in the nonsurvivors.

The mean duration between the diagnosis of severe disease and the introduction of CS was 5.7 ± 5.0 days in the recovered patients, and 8.0 ± 4.6 days in the nonsurvivors. The difference between them was not significant. The mean duration of CS therapy was 77.6 ± 63.2 days in the recovered patients.

Histological examination

Liver histology in 5 patients showed massive necrosis in one nonsurvivor and chronic hepatitis in 4 recovered patients (F2, severe in 1 and F3, severe in 3) (Table 2).

Long-term outcomes of recovered patients

In the 7 recovered patients, LMV was introduced to 4 and ETV to 3, and adefovir was not introduced at all. CS doses were tapered to cessation in all patients.

Discussion

The prognosis of severe acute exacerbation of chronic hepatitis B is very poor if signs of liver failure appear. This is recognized everywhere around the world [1, 18, 19]. The survival rate of patients with fulminant liver failure in HBV carriers was less than 20% without liver transplantation in a Japanese nationwide survey between 1998 and 2003 [18]. No effective therapy other than liver transplantation is

established for severe acute exacerbation of chronic hepatitis B. Therefore, establishing other effective therapies is urgently required for such patients. This has been an important clinical problem in Japan, where a serious shortage of donor livers still remains.

As mentioned above, in our previous study, we reported that the introduction of high-dose CS could reverse deterioration in patients with "clinically severe, life-threatening" exacerbation of chronic hepatitis B, when used in the early stage of the illness [14].

Recently, NAs, which are strongly active against HBV by interfering with HBV reverse transcriptase activity, have been administered in patients with chronic hepatitis B, and dramatic biochemical and histological improvements were achieved. It was proven that NAs could be administered safely even in severe disease [9–13], but the mortality is still high in patients with liver failure. Tsubota et al. [20] reported that LMV monotherapy offered no significant advantage over conventional supportive treatment for rapid progression to hepatic failure, nor did the therapy offer improvement and prolongation of short-term survival in patients with spontaneous severe acute exacerbation of chronic hepatitis B, but they noted that the combination of any effective therapeutic strategies with LMV should be aggressively instituted. Chien et al. [21] reported that LMV failed to prevent death in patients with severe acute exacerbation if it was administered after the serum bilirubin level rose above 20 mg/dl.

HBV DNA is reduced rapidly with the administration of NAs, but improvement in liver function and liver regeneration, is delayed by a few weeks to a few months [10, 14, 15]. During this time-lag phase, excessive immunological reaction may continue, liver cell injury may progress, and liver regeneration may be impaired. If effective therapeutic approaches were to be available in this phase, they would certainly be beneficial for these patients. CS therapy would be a candidate, as it inhibits excessive immune response and prevents cytolysis of infected hepatocytes. Therefore, we defined the criteria of severe disease in 1997, as described above, and after 1997 we treated patients with severe disease with the early initiation of sufficient doses of CS prospectively, and we used a combination of early and sufficient doses of CS and NA after 1999. In our previous studies, we described the significant effect of the combination therapy of CS and NA compared with historical controls [14, 15].

However, CS has not been used for the treatment of chronic hepatitis B because it might enhance the replication of HBV through a steroid-responsive element in the HBV genome [22]. Gregory et al. [23] reported that CS would likely have proved beneficial if treatment had been started "much earlier" in the course of the illness, while CS was equally ineffective in the treatment of severe viral hepatitis by a double-blind, randomized trial. On the other hand, Hansson et al. [24] reported that, in the treatment of fulminant hepatitis B patients with foscarnet, clinical recovery was related to the influence on the immune system rather than an influence on the reduction of HBV DNA.

In our previous study, none of the patients treated with high doses of CS monotherapy showed increased HBV replication during short-term observation periods [14]. In another study, HBV DNA decreased significantly during the first 4-week period with a combination therapy of CS and NA [15]. In these studies, we used a historical control instead of a randomized controlled group, because ethical issues prevent a randomized control study in such life-threatened patients. Therefore, we believe that CS is not contraindicated for the treatment of a specific population of chronic hepatitis B patients.

In the present study, the differences in background parameters on admission, including age, PT activity, T-Bil level, HBV DNA level, AFP level, and HGF level were not significant between the recovered patients and the nonsurvivors. The difference in mean duration between the diagnosis of severe disease and the introduction of CS was also not significant between the two groups. These findings contradicted our expectations that liver function deterioration would be more advanced, liver regeneration would be more impaired, and the timing of introduction of CS would be later in the nonsurvivors than in the recovered patients. The only difference found between the groups on admission was in the presence of fulminant hepatitis.

Regarding the relationship between responses to the therapy and clinical outcomes, improvement of PT activity and decline of HBV DNA between weeks 0 and 2, and between weeks 0 and 4 were found in the recovered patients, and significant improvements of ALT levels between weeks 0 and 2 and between weeks 0 and 4 were found in both groups. The improvement of T-Bil level was not significant during this period in the recovered patients, while the increases of T-Bil level between weeks 0 and 4, and between weeks 2 and 4 were significant in the nonsurvivors.

Taken together, the findings of the present study indicate that patients with fulminant hepatitis on admission, no improvement of PT activity during the first 2 weeks of combined CS and NA treatment, and worsening of T-Bil level during 4 weeks of treatment, especially worsening between week 2 and week 4, could not possibly be salvaged by the combination therapy of CS and NA and would urgently need liver transplantation. This timing of the assessment would be sufficiently early to avoid infectious complications.

We were able to shorten the CS treatment period while monitoring the viral load after we were able to use an NA in combination with the CS from 1999, and recently, the period has become shortened, to 20–30 days.