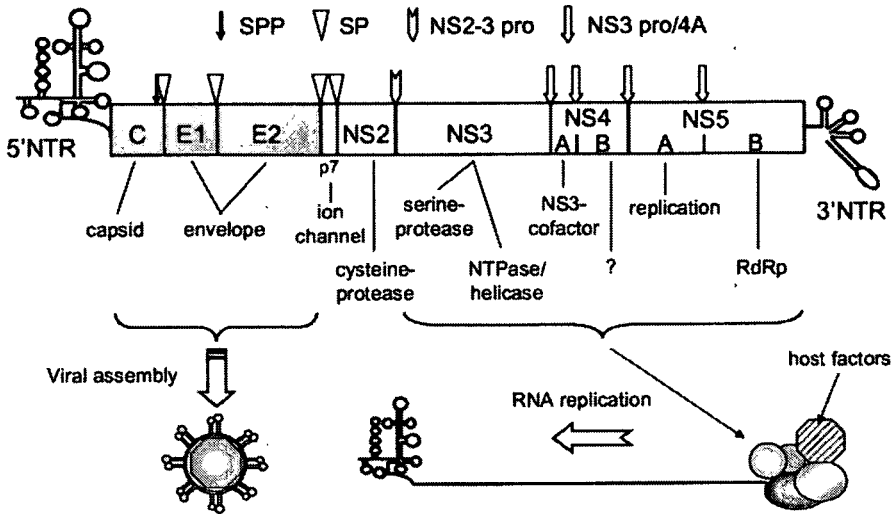

Fig. 1. Hepatitis C virus (HCV) genome organization and polyprotein processing. Posttranslational cleavages by signal peptide peptidase (*SPP*), signal peptidase (*SP*), NS2–NS3 protease (*NS2–3 pro*), and NS3 protease and NS4A complex (*NS3 pro/4A*) lead to the production of functional HCV proteins. *NTR*, nontranslated region

Cell culture systems for HCV research

Although substantial information on HCV protein structure and function has been obtained from the use of a variety of cell culture and in vitro expression systems, for many years, HCV research has been hampered by the restricted host range and the inefficiency of cell culture models for viral infection and propagation. The development of the HCV replicon system, therefore, is a milestone in HCV research and has allowed examination of viral RNA replication in cell culture.[16] Expression systems of heterologous virus genes based on RNA replicons have been established in a variety of positive-strand RNA viruses such as polio virus,[17–20] the alphavirus Semliki Forest virus,[21] Sindbis virus,[22–25] Kunjin virus,[26] human rhinovirus 14,[27] and bovine viral diarrhea virus.[28] In general, advantages of replicon systems are (1) a high level of gene expression and RNA replication, (2) easy construction of recombinants, and (3) a wide permissible host range.

The HCV replicons are typically composed of selectable, bicistronic RNA, with the first cistron containing the HCV 5′ nontranslated region (NTR), which directs translation of the gene encoding the neomycin phosphotransferase, and the second cistron containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus, which directs translation of HCV NS3 through NS5B region, and the 3′ NTR. The prototype subgenomic replicon utilized a particular HCV genotype 1b clone termed Con1. Following transfection of RNA generated by in vitro transcription of the cloned replicon sequences into a human hepatoma cell line Huh-7, antibiotic G418-resistant cells could be obtained in which the subgenomic RNA replicated autonomously. RNA replication was first detected at relatively low frequency, followed by the identification of replicons harboring cell culture-adaptive mutations, which increased the efficiency of replication initiation by several orders of magnitude.[29–31]

Adaptive mutations were found primarily at the N-terminus of the NS3 helicase, in NS4B, and in the center of NS5A, which is upstream of the region putatively involved in IFN sensitivity. Most of the mutations in NS5A are located at highly conserved serine residues and lead to change in the phosphorylation state of NS5A.[32,33] A combination of adaptive mutation in NS3 and NS5A resulted in the highest level of replication of a particular HCV genotype 1b isolate.[31] Later work, however, has indicated that adaptive mutations can arise in most of the viral nonstructural proteins.[34,35] The mechanisms by which adaptive mutations increase RNA replication efficiency are not well understood.

In the last 7 years, a variety of different replicons have been generated, including replicons with reporters or markers such as luciferase and green fluorescent protein, replicons from genotype 1a and 2a, and genome-length dicistronic HCV RNAs (genomic HCV replicons). HCV replicons with reporter genes allow us to execute fast and reproducible screening of large series of compounds for antivirals.[36–38] Huh-7 cells are the most permissive for HCV replicons. However, variability in the permissiveness for replicons has been observed for a given Huh-7 cell pool, and the cells that are able to support efficient replication of the viral genome are enriched during selection such as G418 treatment. A so-called "cured" cell clone, which can be prepared by removing the replicons by treatment with IFN, supports viral replication to a much higher level in many cases and is useful for introducing genome-length HCV RNAs.[39,40]

An HCV genotype 2a replicon with the JFH-1 strain, which was first isolated from the serum of a Japanese patient with fulminant hepatitis C by our group,[41] replicates efficiently in not only Huh-7 cells but also other

hepatocyte-derived cell lines, HepG2 and IMY-N9, and nonhepatocyte-derived cell lines, HeLa and 293.[42–44] Interestingly, the JFH-1 replicon does not require adaptive mutations for replicating in these cell lines, and enormously efficient RNA replication is detected by transient replication assay as well as by colony formation assay with G418 selection,[42] suggesting that the JFH-1 genome can replicate autonomously without the help of drug selection or the requirement of adaptive mutations. This observation laid the basis for a breakthrough in HCV research.

Transfection of the full-length JFH-1 genome into Huh-7 cells leads to the production of HCV particles that are infectious both for naïve cells and for animal models.[45] As a first attempt, an in vitro transcribed full-length JFH-1 RNA was introduced into naïve Huh-7 cells, which is the original cell line used for subgenomic replicon studies. Efficient RNA replication in the transfected cells was detectable by Northern blot analysis, and the viral-enveloped particles, which are spherical structures with an outer diameter of approximately 55 nm, were secreted to the culture medium.[45] Secreted virus was found to be infectious, although at low efficiency, for naïve Huh-7 cells, and its infectivity can be neutralized by anti-CD81 antibody and hepatitis C patients' sera.[45] Subsequently, to increase the infection efficiency, "cured" Huh-7 cell lines such as Huh7.5, Huh7.5.1, and Huh7-Lunet were used. Infectivity of these cured cell lines with JFH-1 became more intense compared with standard Huh-7 cells, and the virus titers released from cells freshly transfected with the JFH-1 genome were markedly increased by continuous passage of the cells carrying persistent replicating viral RNA.[46] Further, chimeric constructs with the core to NS2 region of another genotype 2a clone, J6, improved the infectivity.[47] Thus, this recombinant infectious HCV cell culture system opens avenues of biochemical and genetic studies of the HCV life cycle.

Besides isolating functional molecular clones of HCV that replicate to high levels, to generate a cell culture model that mimics natural host cell environments may be advantageous for improving HCV production systems suitable for studying the virus–host interaction. It is likely that HCV morphogenesis occurs in a complex cellular environment in which host factors may either enhance or reduce the assembly and budding process. Generally, the interaction of viruses with polarized epithelia in the host is one of the key steps in the viral life cycle. A variety of enveloped viruses mature and bud from distinct membrane domains of the host cells.[48–51] We found that a dicistronic HCV genome of genotype 1b supports the production and secretion of infectious HCV particles in two independent three-dimensional (3D) culture systems, the radial-flow bioreactor (RFB) and the thermoreversible gelation polymer (TGP), but not in monolayer cultures, although its productivity is much lower than that observed in the JFH-1 system[52] (Fig. 2). The RFB system was initially aimed at the

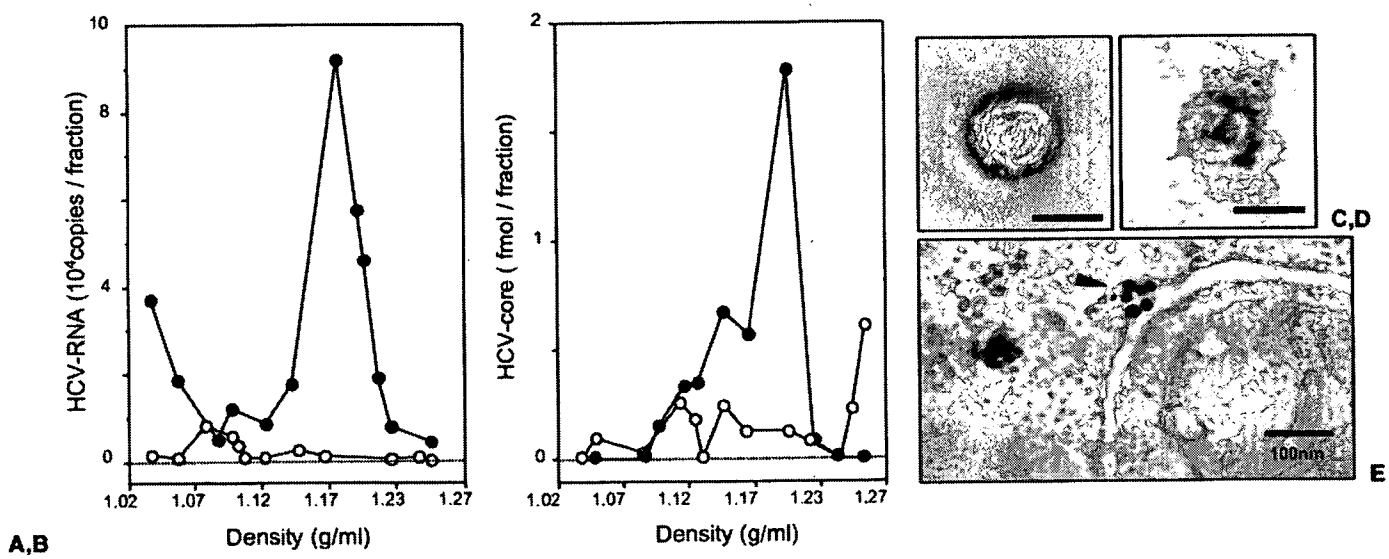

Fig. 2A–E. Production of HCV particles in the three-dimensional, thermoreversible gelatin polymer (TGP) culture of the Huh-7 cell line (RCYM1) carrying genome-length dicistronic HCV RNA of genotype 1b. **A, B** Sucrose density gradient analysis of culture supernatants of RCYM1 cells. The culture supernatants were fractionated and then HCV RNA (**A**) and core protein (**B**) in each fraction were determined by enzyme-linked immunosorbent assay and real-time reverse transcription polymerase chain reaction, respectively. *Closed circles*, TGP culture; *open circles*, monolayer culture. **C, D** Electron microscopy of HCV particles in the supernatants of TGP-cultured RCYM1 cells. **C** Negative staining. **D** Immunogold labeling with an anti-E2 antibody. Gold particles, 5 nm; bars, 50 nm. **E** Silver-intensified immunogold staining with anti-E1 antibody. The *arrowhead* indicates virus-like particles reacting with anti-E1 antibody

development of artificial liver tissue, and the bioreactor column consists of a vertically extended cylindrical matrix through which liquid medium flows continuously from the periphery toward the center of the reactor.[53] In RFB culture, human hepatocyte-derived cells can grow spherically or cubically, and they retain liver functions such as albumin synthesis[53-55] and drug-metabolizing activity mediated by cytochrome P450 3A4.[56] TGP is a chemically synthesized biocompatible polymer which has a sol–gel transition temperature, thus enabling us to culture cells three-dimensionally in the gel phase at 37°C and to harvest them in the sol phase at 4°C, without enzyme digestion.[57] In contrast to other matrix gels made from conventional natural polymers, TGP has several advantages that allow us to investigate the functional characteristics of epithelial cells, their tissue-like morphology, and their potential clinical applications. For example, the use of 3D culture materials other than TGP requires treatment with appropriate digestive enzymes or heating to collect cells grown as spheroids from the culture media, and the matrices may damage the cultured cells to some extent. A 3D culture system based on RFB and TGP, in which human hepatoma cells can assemble into spheroids with potentially polarized morphology, is a valuable tool in studies of HCV morphogenesis.

Translation

The approximately 341-nucleotide (nt)-long 5′ NTR is one of the most conserved regions of the HCV genome, and the secondary structural model, which is also largely conserved, reveals four distinct RNA domains in the region, reflecting its importance in both viral translation and replication.[58-61] The 5′ NTR forms four highly structured domains (domains I–IV), which may be conserved among HCV and related flaviviruses and pestiviruses,[59,60] and it is functionally characterized as an IRES to direct cap-independent translation of the genome.[62,63] To determine the minimal sequence required for HCV IRES-dependent translation, as in the earlier studies of picornaviruses, the bicistronic RNAs in which two reporter protein-coding sequences are separated by an IRES sequence were analyzed. Translation of the upstream reading frame occurs in a 5′ end-dependent fashion, while translation of the downstream reading frame is driven by the IRES element. The IRES comprises nearly the entire 5′ UTR of the genome. There is evidence to suggest that the first 12 to 30 nt of the coding sequence are also important for IRES activity.[64-66] The first 40 nt of the 5′ NTR, which includes a single stem–loop (domain I), is not essential for the translation; the 5′ border of the IRES was mapped between nt 38 and 46.[61,67,68] Domains II and III are relatively more complex and contain multiple stems and loops.[60,69] Domain IV consists of a small stem–loop containing the polyprotein start codon at nt 342 and forms a pseudoknot via base-pairing with a loop in domain III.

Recruitment of the 43S ribosomal complex, containing a small 40S ribosomal subunit, eukaryotic initiation factor (eIF) 3, and a tRNA–eIF2–GTP ternary complex, to mRNA molecules is critical for initiation of eukaryotic protein synthesis. The 40S subunit and eIF3 can bind independently to the HCV IRES.[64,70-72] However, it appears that interaction between IRES RNA and the 40S subunit drives formation of an IRES–40S subunit–eIF3 complex, since HCV IRES RNA demonstrates similar affinity to both the 40S subunit and the 40S–eIF complex.[71] Other cellular factors such as La autoantigen,[73-75] heterogeneous ribonucleoprotein L,[76] poly-C binding protein,[77,78] and pyrimidine tract-binding protein,[79,80] also bind to the IRES element and modulate translation.

Regulation of IRES-dependent translation of HCV is also likely to involve viral factors. We found that the core protein specifically inhibits HCV translation, possibly by binding to a stem–loop IIId domain, particularly a GGG triplet within the hairpin loop structure of the domain, within the IRES (Fig. 3).[79-81] Although a conflicting report has suggested that inhibition of HCV translation is due to an RNA–RNA interaction, rather than to an interaction between RNA and the core protein,[82] later studies support the role of a core protein sequence spanning amino acids (aa) 34–44 in inhibition of viral translation through its interaction with the IRES.[83] Furthermore, the N-terminal 20 residues of the core protein have been shown to selectively inhibit translation mediated by HCV IRES in a cell type-specific manner.[84] We propose a model in which competitive binding of the core protein to the IRES and 40S ribosomal subunit regulates HCV translation.

By analogy with other RNA viruses with IRES-mediated expression, the HCV 5′ NTR has been expected to contain not only determinants for translation but also *cis*-acting elements for RNA replication. Recent studies demonstrated that (1) the sequence upstream of the IRES is essential for viral RNA replication, (2) sequences within the IRES are required for high-level HCV replication, and (3) the stem–loop domain II of the IRES is crucial for the replication.[85]

Polyprotein processing

IRES-mediated translation of the HCV ORF yields a polyprotein precursor that is subsequently processed by cellular and viral proteases into mature structural and nonstructural proteins (Fig. 1). As deduced from the hydrophobicity profile and the dependence on micro-

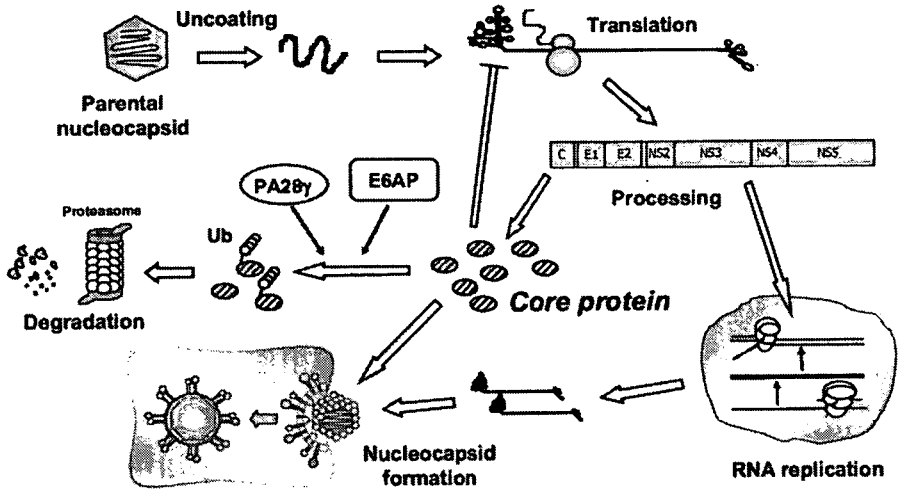

Fig. 3. The role and fate of HCV core protein in the postulated HCV life cycle. See text for further explanation and details

somal membranes, junctions at core/E1, E1/E2, E2/p7, and p7/NS2 are processed by host signal peptidases. For instance, secondary structure analysis of the core protein reveals that all major alpha helices are located in the C-terminal half of the protein. A predicted alpha helix encoded by aa 174–191 is extremely hydrophobic and resembles typical signal peptide sequences. Further posttranslational cleavage close to the C terminus of the core protein takes place, removing the E1 signal sequence by the signal peptide peptidase.[86-89] This peptidase has recently been identified[90] and exhibits protease activity within cellular membranes, resulting in cleavage of peptide bonds in the plane of lipid bilayers.

The viral nonstructural proteins are processed by two viral proteases: processing between NS2 and NS3 is a rapid intramolecular reaction that is accomplished by the NS2–3 protease, which spans NS2 and the N-terminal domain of NS3, whereas the remaining four junctions are cleaved by the serine protease located at the N-terminal 180 residues of NS3 protein. Efficient cleavage at the NS2/3 site requires the 130 C-terminal residues and the first 180 aa of NS3. Recombinant proteins lacking the N-terminal membrane domain of NS2 were found to be enzymatically active, allowing further characterization of this activity.[91,92] Deletion of NS2 from the nonstructural polyprotein did not abolish the replication of HCV RNA in cell cultures, indicating that NS2 is not essential for vial RNA replication.[16,29]

The NS3–NS5B region is processed presumably with the following preferred order of cleavage: NS3/4A→NS5A/5B→NS4A/4B→NS4B/5A.[93-96] Processing at the NS3/4A site is an intramolecular reaction, whereas cleavage at the other sites can be mediated intermolecularly. NS3 is a multifunctional molecule. Besides its N-terminal protease activity, the helicase and nucleotide triphosphatase (NTPase) activities reside in the C-terminal 500 residues of the NS3 protein.[97-101] NS4A functions as a cofactor of the NS3 serine protease and is required for efficient polyprotein processing. There are significant differences in the stability and activity of the NS3 protease in the presence or absence of NS4A. NS3 protein is relatively unstable when expressed in cells in the absence of NS4A.[102] Structural studies by nuclear magnetic resonance and X-ray methods show that the NS3–4A complex has a more highly ordered N-terminal domain and NS4A binding leads the NS3 protease to a rearrangement of the active site triad to a canonical conformation.[103] It has been predicted that the N-terminus of NS4A forms a transmembrane helix, which presumably anchors the NS3–4A complex to the cellular membrane.[104]

RNA replication

HCV is assumed to replicate its genome through the synthesis of a full-length negative-strand RNA. Positive-strand RNA is then produced from the negative-strand template; it is several-fold more abundant than the negative-stranded RNA and is utilized for translation, replication, and packaging into progeny viruses. RNA replication of most RNA viruses involves certain intracellular membrane structures, including the endoplasmic reticulum (ER),[105-107] Golgi,[108] endosomes, and lysosomes.[109] HCV RNA replication is also believed to occur in the cytoplasm of the virus-infected cells.

Although NS5B protein has RNA-dependent RNA polymerase (RdRp) activity in vitro, its recombinant product alone is presumably short of strict template specificity and fidelity, which are essential for viral RNA synthesis. It is highly likely that other viral or host factors are important for conferring proper RNA replication and that the replication complexes (RCs), which are composed of NS5B and additional components re-

quired for modulating polymerase activity, are involved in catalyzing HCV RNA synthesis during the replication process. NS3 is directly involved in RNA synthesis, possibly through its helicase/NTPase activities. The helicase activity is presumed to be involved in unwinding a putative double-stranded replication intermediate or to remove regions of secondary structure so that MS5B RdRp can copy both strands of the viral RNA. It is likely that the NTPase activity is coupled with the helicase function, supplying the energy required for disrupting RNA duplexes. Although little is known about the function of NS4B in the HCV life cycle to date, NS4B protein can induce a membranous web, consisting of small vesicles embedded in a membranous matrix,[110] and it has been reported that the newly synthesized HCV RNA and most of the viral nonstructural proteins occur in these membrane webs or speckle-like structures.[111-113] NS4B may play an important role in the formation of the HCV RNA replication complex.[114] Evidence indicating an involvement of NS5A in viral RNA replication is now accumulating. As described above, a hot spot of the cell culture-adaptive mutations that increase replication efficiency of HCV RNA is located in the central region of NS5A.[29-31] The membrane association of NS5A through its amino-terminal transmembrane domain[115] and the interaction between NS5A and 5B[116] are essential for RNA replication. Several cellular proteins interacting with NS5A have been identified, and human vesicle-associated membrane protein-associated proteins (hVAP-A and -B) are likely to play a key role in RNA replication through the interaction with NS5A.[114,117] The 3′ NTR also contains a significant predicted RNA structure with three distinct domains: a variable region of about 40nt, a variable length poly(U/UC) tract, and a highly conserved, 98-nt 3′ terminal segment (3′X) that putatively forms three stem–loop structures.[118-120] Viral RNA replication was not detected when any of the three putative stem–loop structures within the 3′X region or the entire poly(U/UC) was deleted.[121] The variable region segment also contributes to efficient RNA replication.[122]

Several groups have succeeded in demonstrating the in vitro replication activities of HCV RCs in crude membrane fractions of cells harboring the subgenomic replicons.[123-126] These cell-free systems provide a valuable complement to the in vitro RdRp assays for biochemical dissection of HCV RNA replication and are a useful source for isolation of viral RCs. From the in vitro replication studies, it appears that RNA synthesis can be initiated in the absence of added negative-strand template RNA, suggesting that preinitiated template RNA copurifies with the RC.[124,125,127] Although the newly synthesized single-strand RNA can be used as a template for a further round of double-strand RNA synthesis, no exogenous RNA serves as a template for HCV RC preparation.[125] Added RNA templates might not access the active site of the HCV RCs owing to sequestration by membranes. The HCV RCs contain both positive- and negative-strand RNAs.[124,127] It has also been reported that cell-free replication activity increases at temperatures ranging from 25° to 40°C, and divalent cations (Mn^{2+} and Mg^{2+}) can be used in the reaction.[125,127]

Membrane flotation analysis and a replication assay have shown that viral RNA and proteins are present in detergent-resistant membrane structures, most likely a lipid-raft structure, and RNA replication activity was detected even after treatment with detergent.[123,128] Lipid rafts are cholesterol- and sphingolipid–rich microdomains characterized by detergent insolubility.[129-131] These structures are known to play a critical role in a number of biological processes, such as as regulators and organizing centers of signal transduction and membrane traffic pathways, including virus entry and assembly of, for example, influenza virus,[132-134] human immunodeficiency virus type-1,[27,135,136] Ebola virus, Marburg virus,[137] enterovirus,[138] avian sarcoma and leukosis virus,[139] Coxsackie B virus, adenovirus,[140] measles virus,[16] and respiratory syncytial virus.[141] However, HCV may be the first example of the association of a lipid raft with viral RNA replication.

On the other hand, it has been widely believed that most of the HCV life cycle, including protein processing and genome replication, takes place in the ER, where cholesterol–sphingolipid rafts are not assembled.[110,142-144] Several studies using the replicon system have indicated that the nonstructural proteins are associated with the ER.[143,145] Nevertheless, it is still possible that HCV nonstructural proteins synthesized at the ER relocalize to lipid-raft membranes when they are actively engaged in RNA replication. It has been shown by membrane separation analysis that HCV nonstructural proteins are present both in the ER and the Golgi, but the activity of viral RNA replication was detected mainly in the Golgi fraction.[123,146] Further studies to elucidate where and how the HCV genome replicates in infected cells are needed.

Viral assembly

The assembly of HCV and the virion structure remains largely unknown. By analogy with related viruses, the mature HCV virion presumably possesses a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. HCV virions are thought to have a diameter of 40–70nm.[147,148] These observations were recently confirmed by immunoelectron microscopy of infectious HCV particles produced in cell cultures.[45,52] It has been reported that HCV circulates in various forms

in the sera of infected hosts, for example, as (1) free mature virions, (2) virions bound to low-density lipoproteins and very low density lipoproteins, (3) virions bound to immunoglobulins, and (4) nonenveloped nucleocapsids, which exhibit physicochemical and antigenic properties.[147-150]

The HCV structural proteins (core, E1, and E2) are located in the N-terminal one-third of the precursor polyprotein (Fig. 1). A crucial function of the core protein, which is derived from the N-terminus of the viral polyprotein, is assembly of the viral nucleocapsid. The aa sequence of this protein is well conserved among different HCV strains, compared with other HCV proteins. The N-terminal domain of the core protein is highly basic, while its C-terminus is hydrophobic. When expressed in mammalian cells and transgenic mice, the core protein is found on membranes of the ER, on the surface of lipid droplets, on the mitochondrial outer membrane, and, to some extent, in the nucleus.[151-156] The core protein is likely multifunctional and is not only involved in formation of the HCV virion but also has a number of regulatory functions, including modulation of lipid metabolism and hepatocarcinogenesis.[153,157-159] The envelope proteins E1 and E2 are extensively glycosylated and have an apparent molecular weight of 30–35 and 70–75 kDa, respectively. Predictive algorithms and genetic analyses of deletion mutants and glycosylation-site variants of the E1 protein suggest that E1 can adopt two topologies in the ER membrane: the conventional type I membrane topology and a polytopic topology in which the protein spans the ER membrane twice with an intervening cytoplasmic loop.[160] E1 and E2 proteins form a noncovalent complex, which is believed to be the building block of the viral envelope.

Several expression systems have been used to investigate HCV capsid assembly using mammalian, insect, yeast, bacteria, and reticulocyte lysates, as well as purified recombinant proteins.[148,161-170] The results suggest that immunogenic nucleocapsid-like particles are heterologous in size and range from 30 to 80 nm in diameter. The N-terminal half of the core protein is important for nucleocapsid formation.[163,169,170] HCV capsid formation occurs in the presence or absence of ER-derived membrane, which supports cleavage of the signal peptide at the C-terminus.[170]

Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. In fact, study of a recombinant mature core protein has shown it to exist as a large multimer in solution under physiological conditions, within which stable secondary structures have been observed.[171] Studies using yeast two-hybrid systems have identified a potential homotypic interaction domain within the N-terminal region of the core protein (aa 1–115 or –122), with particular emphasis on the region encompassing aa 82–102.[172,173] However, other studies have identified two C-terminal regions, extending from aa 123 to 191 and from 125 to 179, as responsible for self-interaction. Furthermore, Pro substitution within these C-terminal regions has been observed to abolish core protein self-interaction.[171,174] Circular dichroism spectroscopy has further shown that a Trp-rich region spanning aa 76–113 is largely solvent-exposed and unlikely to play a role in multimerization.[171] Recently, our group demonstrated that self-oligomerization of the core protein is promoted by aa 72–91 in the core.[160]

Once a HCV nucleocapsid is formed in the cytoplasm, it acquires an envelope as it buds through intracellular membranes. Interactions between the core and E1/E2 proteins are considered to determine viral morphology. Expression of HCV structural proteins using recombinant virus vectors has led to successful generation of virus-like particles with similar ultrastructural properties to HCV virions. Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the ER has been noted.[161,175,176] Mapping studies to determine the nature of interaction between core and E1 proteins have demonstrated the importance of C-terminal regions in this interaction.[177,178] Since corresponding sequences are not well conserved among various HCV isolates, interactions between core and E1 proteins might depend more on hydrophobicity than on specific sequences. By contrast, it has been shown that interaction between the self-oligomerized HCV core and the E1 glycoprotein is mediated through the cytoplasmic loop present in a polytopic form of the E1 protein.[160]

Implication of the ubiquitin–proteasome pathway in core protein maturation

The ubiquitin–proteasome pathway is the major route by which selective protein degradation occurs in eukaryotic cells and is now emerging as an essential mechanism of cellular regulation.[179,180] This pathway is also involved in the posttranslational regulation of the core protein.[158,181-183] We have reported that processing at the carboxyl-terminal hydrophobic domain of the core protein leads to its efficient polyubiquitylation and proteasomal degradation.[181] Recently, our group identified the ubiquitin ligase E6AP as an HCV core-binding protein and showed that E6AP enhances ubiquitylation and degradation of the mature as well as the carboxyl-terminally truncated core proteins, and that the core protein produced from infectious HCV is degraded via an E6AP-dependent pathway (Fig. 3).[183] E6AP, the prototype of HECT domain ubiquitin ligases,[184] was initially identified as the cellular factor that stimulates ubiquitin-dependent degradation of the tumor suppres-

sor p53 in conjunction with E6 protein of cancer-associated human papillomavirus types 16 and 18.[185,186] Exogenous expression of E6AP reduces intracellular core protein levels and supernatant viral infectivity in infected cell cultures. Knockdown of exogenous E6AP by siRNA increases intracellular core protein levels and virus titers in the culture supernatants. The core protein interacts with E6AP through the aa 58–71 region of the core, which is highly conserved among all HCV genotypes, suggesting that E6AP-dependent degradation of the core protein is common to a variety of HCV isolates and plays a critical role in the HCV life cycle or viral pathogenesis.

A role for the proteasome activator PA28γ core-binding protein in degradation of the core protein has also been demonstrated (Fig. 3).[158,182] Overexpression of PA28γ promotes proteolysis of the core protein. PA28γ predominates in the nucleus and forms a homopolymer, which associates with the 20S proteasome,[187] thereby enhancing proteasomal activity.[188] Both nuclear retention and core protein stability are regulated via a PA28γ-dependent pathway.

In eukaryotic cells, targeted protein degradation is increasingly understood to be an important mechanism by which cells regulate levels of specific proteins, and thereby regulate their function. The core protein is believed to play a key role in viral replication and pathogenesis since it forms the viral particle and regulates a number of host cell functions. Although the biological significance of ubiquitylation and proteasomal degradation of the core protein is not fully understood, E6AP possibly affects the production of HCV particles through controlling the amount of core protein (Fig. 3). This mechanism may contribute to virus persistence by maintaining a (moderately) low level of the viral nucleocapsid. The E6AP binding domain within the core protein resides in the region that is considered to be important for binding to the viral RNA and several host factors.[189] These factors may affect the interaction between the core and E6AP, resulting in control of E6AP-dependent core degradation. A recent study demonstrated that a knockdown of the *PA28γ* gene induces the accumulation of the core protein in the nucleus of hepatocytes of HCV core gene-transgenic mice and disrupts development of both hepatic steatosis and hepatocellular carcinoma.[158] Upregulation of several genes related to fatty acid biosynthesis and lipid homeostasis by the core protein was observed in the cells and the mouse liver in the PA28γ-dependent manner. Thus, it is likely that PA28γ plays an important role in the development of liver pathology induced by HCV infection.

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コンセンサス

肝疾患

B型肝炎・C型肝炎の治療

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C型肝炎治療のコンセンサス

2 Peg-IFN/ribavirin 併用療法

4 抗ウイルス療法の安全性と治療効果

荒瀬康司，鈴木文孝，熊田博光

ポイント

コンセンサス　HCV RNAが100 KIU/ml以上の高ウイルス例に対しては，Peg-IFN/ribavirin 併用療法が第一選択である．

コンセンサス　Peg-IFN/ribavirin 併用療法では，ウイルス，宿主，治療の3面よりウイルス排除の可能性を予測して治療導入することが望ましい．

コンセンサス　トランスアミナーゼ正常例に対してもPeg-IFN/ribavirin 併用療法はトランスアミナーゼ異常例と同様に治療効果が期待できる．

要検討　Peg-IFN/ribavirin 併用療法中，HCV RNA が陽性であってもトランスアミナーゼが正常であれば治療終了後のトランスアミナーゼ正常化を期待できる．

要検討　Peg-IFN/ribavirin 併用療法開始後，HCV RNAの陰性化が12週以降の場合には，72週など長期的な治療を行うことによりウイルス排除率を改善できる．

コンセンサス　Peg-IFN/ribavirin 併用においては，① 高齢者（65ないし70歳以上），② 糖尿病・高血圧合併症例，③ 血球減少例などはとくに副作用による脱落に注意が必要である．

はじめに

C型慢性肝炎に対する抗ウイルス療法は，近年飛躍的な進歩がみられている．1998年ヨーロッパおよび米国よりinterferon（IFN）/ribavirin 併用療法の治療効果が発表されてより，もっとも抗ウイルス作用の強い療法として広まってきた．さらにIFNにポリエチレングリコール（PEG）を共有結合させたPeg-IFNが登場し，2004年12月よりは，genotype 1型高ウイルス量に対しては，Peg-IFN/ribavirin 併用療法での1年間投与の保険認可がなされてきた．さらに2005年12月よりは，genotype 1bかつ高ウイルス量以外の症例に対して，Peg-IFN/ribavirin 併用療法での24週投与の治療が認可されるようになってきた．

そこで今回は，Peg-IFN/ribavirin 併用療法における安全性とその治療効果などにつき，現在の動向を記していく．

Ⅰ. 治療効果

IFN/ribavirin 併用療法は，その副作用によりしばしば脱落が認められるが，全体でいうと，治療効果は以前の IFN 単独療法に比し，飛躍的に伸びてきた．とくにヨーロッパおよび米国では IFN/ribavirin 併用療法の効果をみるために IFN α-2b 3 MU 週 3 回投与に ribavirin 1 日 1,000 ～ 1,200 mg を併用した大規模な無作為二重盲検試験が行われた[1),2)]．48 週投与での結果は表 1 のようであった．なお治療効果を sustained virologic response（SVR）；治療終了 24 週時点での HCV RNA 陰性化で示した．

さらに，genotype 1 の高ウイルス量例に対する Peg-IFN/ribavirin 併用療法 48 週投与の国内臨床試験ではウイルス排除率は 254 例中 121 例（47.6%）との報告が出されており，従来の報告に比し，高いウイルス排除率であった．

しかしながら，副作用により治療を断念せざるをえない場合には，治療効果が著しく減少してしまうため，その副作用の発症には注意が必要となる．また，副作用があったとしても早めに減量して，IFN を許定量の 80% 以上を使えば治療効果が良好であるとの報告も出されている[3)]．

次に，genotype 2 の高ウイルス量症例に対して，IFN 単独ではウイルス排除率が 24 週投与においては 50% 前後であったが，Peg-IFN α-2b と ribavirin の併用療法 24 週投与では，ウイルス排除率は国内臨床試験では 88% であった．

その後も ribavirin 併用療法での効果が良好との多くの報告がなされており，現時点では高ウイルス例に対しては，Peg-IFN/ribavirin 併用療法が第一選択と考えてよいと思われる[4)～16)]．

Ⅱ. 治療効果に寄与する要因

Peg-IFN/ribavirin 併用療法を行った場合に，根本的目標は，ウイルス排除である．しかしながら，すべての症例でウイルスが排除できるわけではない．ウイルス排除に寄与する要因につき検討したところ，それは大きく，ウイルス側要因，宿主側要因，治療側要因の三つに分けて考えられる．

ウイルス側要因のなかでは，ウイルスのコア蛋白において，70 番目のアミノ酸がグリシンからアスパラギン酸，91 番目のアミノ酸がロイシンからメチオニンに変異している場合には治療効果が不良であるということが判明してきた[17)]．また，ウイルスの量に関しては，Peg-IFN/ribavirin 併用療法ではウイルスが非常に多い量であっても，少ない量の群に比し有意差はないというような結果であった．ただし，治療開始から 2 週間以内に HCV-RNA 量が 1/100 以下となった症例では効果は良好であるとの報告がなされている[18)]．また，宿主側要因では LDL コレステロールが低すぎるような症例，高齢症例，女性症例，あるいは合併症として糖尿病をもっているような症例では治療効果が不良であるとの報告もある．さらに治療要因では，ribavirin の血中の濃度が低すぎる場合には，治療効果が不良である[7),8)]．したがって，これらの要因を事前にチェックあるいは，修正できるものは修正してから治療に入る必要性もあると考えられる．

表 1　IFN/ribavirin 併用療法の効果

Author	治療法	症例数	SVR(総数)(%)	SVR(genotype 1 のみ)(%)
Poynard（ヨーロッパ）	IFN 単独	281	19	
	Ribavirin 併用療法	281	43	
McHutchison（米国）	IFN 単独	225	13	7
	Ribavirin 併用療法	228	38	28

Ⅲ. トランスアミナーゼ正常例に対するIFN投与

トランスアミナーゼ正常例に対しては，従来IFN投与は行わないことが多かったが，トランスアミナーゼが正常であっても年率5～10%前後でトランスアミナーゼの上昇がみられ，また長期的にみると70～80歳になって肝癌が発症する例も存在する．また，70歳代になって症状が進展した際に治療を開始しようとしても，治療を行うには高齢化しており，治療を断念せざるをえない場合も多い．

そこで50歳代ないし60歳前後の，抗ウイルス治療に十分耐えられる年齢で治療が導入されることが考慮されるようになってきた．その効果であるが，Peg-IFN/ribavirin併用療法は，トランスアミナーゼ異常例と比べてほぼ同等の治療効果が期待されるとの報告が多い[19]．したがって，高齢化に至る以前にPeg-IFN/ribavirin併用療法を行うのも一法と考えられる．

Ⅳ. トランスアミナーゼ正常化を目指したIFN/ribavirin効果

Peg-IFN/ribavirin併用療法は，1b高ウイルス量症例では治療開始より3カ月時点で約50%，6カ月の時点で約75%の症例で血清学的なウイルスの排除がみられる．逆にいえば約25%の症例ではHCV-RNAが陽性のままであるということであり，これらの症例に対し治療を継続するか否かということが一つの問題である．

現時点ではPeg-IFN/ribavirin併用療法開始24週の時点で，ウイルスが陰性化しないような症例でも，トランスアミナーゼの正常化がみられていればこのIFN服用方法を継続し，48週までもっていくと治療終了後のトランスアミナーゼの正常化が長期に持続することが判明してきた．

Ⅴ. Peg-IFN/ribavirin併用の長期投与での効果

Peg-IFN/ribavirin併用療法は，genotype 1bの高ウイルス量例であっても12週未満で血清ウイルスRNAが陰性化すれば，48週投与でも十分ウイルス排除が期待できる．すなわち，国内第Ⅱ相臨床試験では12週までに血清HCV-RNAの陰性化がみられた例ではSVRは74.3%（202/272）と高率であった．しかしながら，12週を超えて24週でHCV-RNAが陰性化したような症例では，48週で治療を中止するとSVRは39%（32/82）にとどまった．このようにHCV-RNAが12週を超えてから陰性化した症例に対し併用療法を長期的に使用した場合に，治療効果を上げられるかが問題である．

当院では現時点では，12週を超えて24週でHCV-RNAが陰性化したような症例で，72週まで治療を継続した際のSVRは63.6%（7/11）にとどまった．このように，HCV-RNAが12週を超えて陰性化したような症例には，72週などの，より長期的な治療継続が必要と思われる．

Ⅵ. 安全性

IFNは生理活性を有するサイトカインに属し，C型肝炎ウイルス量の増殖を抑えると同時に生体内で種々の副作用を呈する．IFN単独療法の際も副作用がみられ，その投与中に数%～10%台の症例が副作用により治療を断念せざるをえない状況であった[19]．一方，Peg-IFN/ribavirin併用療法においては，ribavirinが加わったためにより重篤な副作用がしばしば認められる．その代表的副作用を表2にまとめて示す．

Peg-IFN/ribavirin併用療法はIFN単独療法に比し，貧血をきたしやすく，高齢者での脱落例が多く，皮疹・間質性肺炎などが多い．さらにこの併用療法においては，糖尿病あるいは高血圧の合併症がある高齢の患者に治療を行った場合には，脳出血などの合併に懸念する必要がある[20)~22)]．また，一部の症例では，KL6の増加が認めら

表2　IFN(ないし Peg-IFN)/ribavirin 併用療法での副作用の程度

IFN α-2b ないし Peg-IFN α-2b と ribavirin の投与を受けると多くの患者で副作用が認められる．代表的副作用には次のようなものがある．(国内臨床試験時の 269 例のデータ)

1. インフルエンザ様症状：発熱(95.9%)，全身倦怠感(93.7%)，頭痛・頭重感(90%)，悪寒(32%)など
2. 精神神経系：不眠(66.5%)，めまい(40.5%)，抑うつ(13.4%)，易刺激性(12.6%)，耳鳴(6.3%)，注意力障害(5.9%)，気分不快(5.6%)，5%未満の副作用としては眠気，異常感，気分低下，健忘，不安，耳閉，意識障害，難聴，感情不安定，失神，心身症，そう状態，圧迫感，過眠，活動低下，構語障害，自殺企図，自律神経失調，神経過敏，大脳萎縮，知覚過敏・減退，聴覚過敏，判断力低下，片頭痛
3. 血液：リンパ球減少(96.7%)，白血球減少(96.7%)，顆粒球減少(73.4%)，好中球減少(88.8%)，ヘモグロビン減少(87.4%)，赤血球減少(81%)，ヘマトクリット減少(79.7%)，網状赤血球増多(72.5%)，血小板減少(46.1%)，リンパ球増多(33.1%)，好塩基球増多(27.1%)，網状赤血球減少(25.3%)，好酸球増多(19.7%)，好中球増多(18.2%)，貧血(8.2%)，5%未満の副作用としては白血球増多，赤血球増多，ヘマトクリット増加，ヘモグロビン増加，骨髄抑制，出血傾向
4. 肝臓：ビリルビン血症(41.3%)，γ GTP 上昇(17.1%)，GOT(AST)上昇(16.7%)，LDH 上昇(16.0%)，GPT(ALT)上昇(14.9%)，5%未満の副作用として AL-P 上昇，脂肪肝，肝血管腫
5. 腎臓：膀胱炎(6.3%)，頻尿(5.2%)，5%未満の副作用として排尿障害，血尿，蛋白尿，尿路結石，濃縮尿，BUN・クレアチニン上昇，クレアチニン減少，神経因性膀胱，腎結石，尿失禁，尿変色，膀胱不快感
6. 循環器：頻脈(21.2%)，潮紅(20.1%)，胸痛(12.3%)，浮腫(四肢・顔面)(5.9%)，血圧上昇(5.2%)，5%未満の副作用として末梢性虚血，血圧低下，心房細動，静脈瘤，蒼白，虚血性脳血管障害
7. 消化器：食欲不振(84%)，悪心・嘔吐(58.4%)，腹痛(55.4%)，下痢(36.8%)，口内・口唇炎(26.8%)，便秘(22.7%)，胃不快感(16.7%)，口渇(15.6%)，歯髄・歯周・歯肉炎(8.9%)，消化不良(8.6%)，歯痛(7.1%)，腹部不快感(6.3%)，舌炎(5.9%)，5%未満の副作用として胃炎，痔核，口腔内不快感，腸管機能異常，おくび，血便，鼓腸放屁，歯の異常，腸炎，排便異常など
8. 皮膚：脱毛(68%)，瘙痒(60.2%)，発疹(56.9%)，皮膚乾燥(19.7%)，湿疹(15.6%)，紅斑(10.8%)，湿疹(16.2%)，白癬(6.3%)，皮膚炎(5.9%)，5%未満の副作用として爪の異常，紫斑，蕁麻疹，接触性皮膚炎，過角化，皮膚潰瘍など
9. 神経・筋：関節痛(75.8%)，筋肉痛(69.9%)，背部・腰部痛(38.3%)，感覚異常(17.1%)，筋痙直(14.5%)，四肢痛(7.4%)，5%未満の副作用として頸部痛，神経痛，肋骨痛，振戦，疼痛，筋硬直，関節炎，腫脹など
10. 呼吸器：上気道炎(47.2%)，咳嗽(29%)，呼吸困難(25.7%)，喀痰増加(12.3%)，鼻出血(11.2%)，鼻漏(7.8%)，5%未満の副作用として鼻炎，鼻閉，くしゃみ，鼻乾燥，嗄声，鼻道刺激感，扁桃炎，咽頭紅斑，咽頭腫脹など
11. 眼：角膜・結膜炎(9.3%)，眼痛(6.7%)，霧視(6.7%)，網膜滲出物(6.3%)，網膜出血(5.9%)，眼精疲労(5.2%)，5%未満の副作用として眼瘙痒感，眼充血，視覚異常，網膜症，眼の違和感，眼瞼炎，眼瞼浮腫，硝子体浮遊物，眼瞼紅斑，視力低下，麦粒腫，網膜裂孔，羞明など
12. 投与部位(注射部反応)：紅斑(40.1%)，瘙痒(24.5%)，発疹(8.2%)，疼痛(7.8%)，腫脹(5.6%)，5%未満の副作用として熱感，炎症，硬結，出血，皮膚炎など
13. その他：甲状腺機能異常(38.3%)，味覚障害(26.8%)，体重減少(21.2%)，CRP 上昇(19.3%)，疲労(11.2%)，感染症(10.8%)，多汗(7.4%)，高血糖(5.9%)，5%未満の副作用として花粉症，高尿酸血症，高蛋白血症，嗅覚異常，リンパ節炎，処置後局所反応，単純疱疹，外耳炎，中耳炎，血中コレステロール増加，月経異常，創傷治癒遅延，帯状疱疹，低アルブミン血症，冷汗など

表3　IFN(ないしPeg-IFN)/ribavirin併用療法での重大な副作用と対策

1. 間質性肺炎(5%未満)，肺線維症，肺水腫(頻度不明)：咳，呼吸困難などが現れた場合には，ただちに担当医に連絡させる．
2. 抑うつ(5%以上)，自殺企図(1%未満)：不眠，不安，焦燥感が現れた場合には，ただちに担当医に連絡させる．
3. 貧血〔赤血球減少250万/mm^3未満(5%未満)，ヘモグロビン減少8 g/dl未満(5%未満)，ヘモグロビン減少8以上9.5 g/dl未満(5%以上)，ヘモグロビン減少9.5以上11 g/dl未満(5%以上)〕：定期的に血液検査を施行する．
4. 無顆粒球症(頻度不明)，白血球減少2,000/mm^3未満(5%以上)，顆粒球減少1,000/mm^3未満(62.1%)：定期的に血液検査を施行する．
5. 血小板減少50,000/mm^3未満(5%未満)：定期的に血液検査を施行する．
6. 再生不良性貧血(頻度不明)，汎血球減少(頻度不明)
7. 意識障害(5%未満)，痙攣，見当識障害，せん妄，錯乱，幻覚，妄想，昏迷，攻撃的行動，統合失調症様症状，痴呆様症状[とくに高齢者]，興奮(頻度不明)，失神，そう状態，難聴(5%未満)
8. 自己免疫現象(頻度不明)によると思われる症状，徴候の増悪または発症など〔甲状腺機能異常，肝炎，溶血性貧血，潰瘍性大腸炎，関節リウマチ，インスリン依存型糖尿病(IDDM)の増悪または発症など〕
9. 糖尿病(5%未満)
10. 重篤な肝障害(頻度不明)
11. 急性腎不全などの重篤な腎障害(頻度不明)
12. ショック(頻度不明)
13. 心筋症，心不全，心筋梗塞，狭心症(頻度不明)
14. 不整脈(5%未満)：心室性不整脈，高度房室ブロック，洞停止，高度徐脈，心房細動など
15. 消化管出血(下血・血便等)(5%未満)，消化性潰瘍(頻度不明)，小腸潰瘍，虚血性大腸炎(5%未満)
16. 呼吸困難，喀痰増加(5%以上)
17. 脳出血(頻度不明)
18. 脳梗塞(頻度不明)
19. 敗血症(頻度不明)
20. 網膜症(5%未満)
21. 皮膚粘膜眼症候群(Stevens-Johnson症候群)，中毒性表皮壊死症(Lyell症候群)(頻度不明)
22. 横紋筋融解症(頻度不明)

れている．併用療法にみられる重篤な副作用とその対策を表3に示している．

これら副作用でとくに注目すべきは，65歳以上でヘモグロビン（Hb）が13 g/dl以下で治療開始した場合には，80%の例でPeg-IFNないしribavirinの減量が必要であったと報告されている．Iwasakiら[3]も併用療法は，高齢とくに60歳以上ではその脱落率が高いと報告している．

いずれにせよ，高齢で糖尿病，高血圧など合併症があるような症例，貧血などのあるような症例では，治療に注意する必要がある．

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