MOLECULAR INVESTIGATION OF HEPATITIS VIRUSES CAUSING PERSISTENT INFECTION

Ph.D. Thesis

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Introduction

Hepatitis viruses are infectious agents, whose primary site of replication is the liver. Hepatitis A to E viruses are sometimes called classic hepatitis viruses, of which only hepatitis B and C viruses can cause chronic infections by themselves. Most cases of viral hepatitis today are caused by these two viruses. Advances in molecular techniques in the last few decades have led to the discoveries of several previously unknown viruses, which may be the causative agents behind some of the cases of viral hepatitis with unknown etiology. These recently discovered viruses include the Anelloviruses and hepatitis G virus (GB virus C).

Hepatitis B virus

Hepatitis B virus (HBV) is classified into the *Ortohepadnavirus* genus of the *Hepadnaviridae* family. The complete virions are spherical and surrounded by a lipid envelope. The viral genome is partially double-stranded circular DNA. Target cells of HBV are hepatocytes. The unique viral replication method includes the formation of an RNA intermediate. HBV is transmitted by blood and blood products and sexually. Safe and effective recombinant vaccine against HBV is available About 400 million people in the world are chronically infected with hepatitis B. The prevalence of HBV infection in Hungary is low. The risk of developing chronicity depends on the age at primary infection: almost all children infected at birth become chronic virus carriers. HBV is non-cytopathic, liver damage is caused by the virus specific T-cell response. A small percentage of acute infections become fulminant, which may be lethal. Chronic HBV carriers may develop cirrhosis and hepatocellular carcinoma. Antiviral therapy of HBV includes pegylated interferon and nucleotide and nucleoside analogues.

Hepatitis C virus

Hepatitis C virus (HVC) is the member of the *Hepacivirus* genus of the *Flaviviridae* family. The spherical virions are enveloped. Its genome is single-stranded RNA with positive polarity and most of it is highly variable. At present HCV variants are grouped into six genotypes and a large number of subtypes. The primary target cells of HCV are hepatocytes, but it infects other cell types as well. The virus is mainly transmitted parenterally. According to estimates at least 170 million people carry HCV worldwide. In developed countries most new cases of acute HCV infection are a consequence of intravenous drug use. Most primary infections are asymptomatic, and about 70-85% of the patients become chronic carriers. HCV

is noncytopathic, but chronic inflammation of the liver may lead to fibrosis, cirrhosis and hepatocellular carcinoma. The best results in antiviral therapy for HCV are achieved using the combination of pegylated interferon α and ribavirin. The efficiency of the therapy greatly depends of the viral genotype.

Hepatitis G virus / GB virus C

GBV-C is a member of the *Flaviviridae* family, but it's not classified into any of the existing genera. The virus was discovered by two groups independently in 1995. Since then it has been shown that HGV/GBV-C is primarily lymphotropic, and the early evidence supporting its hepatotropism were artefacts. The genome of GBV-C is single-stranded RNA with positive polarity, there are 6 known genotypes. HGV-C is mainly transmitted parenterally, but sexual and perinatal transmission have also been reported. Infection with the virus is probably asymptomatic and the virus may persist for years. Studies show that there is an interference between GBV-C and HIV, and some results even suggest that coinfection with GBV-C in HIV positive patients may be beneficial for their survival. During the adaptive immune response neutralizing antibodies against envelope proteins may be produced and concurrently the virus is cleared from the serum.

Anelloviruses

The *Anelloviridae* family is a group of viruses showing great diversity. The first member of the virus family was discovered in 1997. Today these viruses are grouped into 9 genera, which include Torque Teno viruses (TTV), Torque Teno minivirus (TTMV), Torque Teno midivirus (TTMDV) and animal TTVs. Their common characteristic is the single-stranded circular DNA genome with negative polarity. The genome structure is similar in all genera, but its size and sequence vary greatly. The replication intermediate of TTVs have been detected in many organs and tissues, but the main sites of replication are lymphoid cells and hepatocytes. The infection may be transmitted enterally, parenterally and sexually. Anelloviruses are widespread, their prevalence is around 90% even in the healthy population. Coinfections and superinfections are also common. Some of the infections are transient, but many become persistent. Pathogenicity of Anelloviruses is still controversial. Most infections are asymptomatic, but some TTV variants are suspected to play a part in hepatitis and other diseases. Infection with swine TTVs (TTSuV) are common in pigs worldwide, but they have not been shown to cause disease in the infected animals. It is possible however, that the consumption of the meat of these animals may carry the risk of zoonotic transmission of TTSuV.

Aims

In the past decades molecular methods in virology have become very sophisticated and widespread, which led to the discoveries of several viruses and new types of assays. In the course of this work we used molecular techniques to study classical and recently discovered hepatitis viruses causing persistent infections.

In case of newly discovered hepatitis viruses our aim was to determine their prevalence in the Hungarian population and to identify their dominant genotypes. Prevalence of HGV/GBV-C infection was measured in the healthy population and in a multirisk group. The frequency of infection with TT viruses belonging to the 3rd genogroup was determined among the health care workers of a hospital. Swine TTVs were detected in samples of weaned piglets of a Hungarian piggery. The detected viruses were also genotyped.

During our work with hepatitis B and C we investigated an intrafamilial outbreak and three outbreaks associated with health care. Our aims were to provide molecular evidence for the common origin of the infections and also tried to identify their source, if possible.

Methods

All samples (serum or tissue) were stored frozen at -20°C until the analysis. Viral nucleic acids were extracted by phenol-chloroform and spin column methods. Extracted RNA was reverse transcribed into cDNA using murine leukaemia virus reverse transcriptase in the presence of random hexamers. Viral genomes in the DNA and cDNA solutions were detected by polymerase chain reaction with newly designed or previously published primers. PCR products were visualized under UV illumination after agarose gel electrophoresis and ethidium bromide staining.

Nucleotide sequencing of PCR products was carried out to identify viral genotypes and to determine the genetic relatedness of the detected strains. When necessary, PCR products were cloned into plasmid vectors, and then plasmids purified using phenol-chloroform or spin column extraction methods. Other PCR products were directly sequenced. Sequencing reactions were performed using flourescein labelled primers or fluorescently labelled dideoxy nucleotide triphosphates. In the latter case the reaction was cyclic.

The obtained sequences were manually checked and cut to equal length, then they were aligned using a multiple alignment program. Phylogenetic trees were constructed using different software. Reference sequences for the analysis representing different known

genotypes and subtypes of the viruses were obtained from GenBank. In case of HBV and HCV outbreaks viral sequences of Hungarian virus carriers were also used as local controls. Most phylogenetic trees were constructed using the neighbor-joining method and Kimura's 2-parameter substitution model. To achieve better resolution, phylogenetic analysis of the viral sequences from the HCV outbreak at the oncology ward was carried out using the maximum likelihood method and the General Time Reversible substitution model.

Results and discussion

Prevalence and genotypes of HGV/GBV-C in Hungary

We detected HGV/GBV-C in the serum samples of 124 healthy persons from Budapest and 75 residents of the housing estate „Dzsumbuj", who were considered a multirisk group for acquiring parenterally transmitted infections. Frequency of intravenous drug use was 28% among this group, and prostitution and promiscuity were also common. Since the virus is mainly transmitted by blood, higher prevalence was expected. Some of the detected viruses were genotyped using type-specific PCR primers or by sequencing and phylogenetic analysis of parts of the genome.

The results showed that the frequency of infection in the healthy group was 8.1%. This is similar to, though slightly higher than that in other European countries. Prevalence of infection with HGV/GBV-C was much higher in the multirisk group (9/75, 12%). In both groups the ratio of virus carriers was highest in the age groups that are sexually the most active. This supports the significance of sexual transmission of HGV/GBV-C. All detected HGV/GBV-C viruses were identified as genotype 2. According to previous publications this genotype is dominant in Europe [Takács et al., 2002, Dencs & Sebestyén, 2007].

Prevalence and genotypes of TT viruses in Hungarian health care workers

We determined the prevalence of genogroup 3 TT viruses in the among 185 health care workers of a Hungarian hospital using genogroup specific primers. Genotype specific primers were used to detect SENV-D and SENV-H genotypes. Serum samples of 40 healthy persons were used as controls. Some of the detected viruses were also genotyped by sequencing the obtained PCR products. TTV variants were detected in 9 serum samples of a TTV carrier health care worker taken over a period of 15 years.

TTV infection was very common in both the health care workers and in the control group (70.3% and 77.5%). Prevalence increased with age, but it as high even in the youngest age group (24%). SENV-H was similarly frequent in the two groups tested (25.9% and 35%), but SENV-D could be detected in significantly more of the health care workers (22.7% and 7.5%), but the difference may have been due to the low number of samples. The ratio of TT virus carriers was high in all hospital units, which suggests there's no association between TTV infection and health care work. Presumably infection with genogroup 3 TT viruses is a common natural phenomenon. TTV genotype 16 was found to be dominant. Besides genotypes already published in GenBank we also found a TTV strain, which may represent a previously undescribed genotype.

At least 6 different group 3 TTV strains could be detected in the serum samples of the TTV carrier health care worker. Some strains could be detected in samples taken several years apart, showing they persisted for years. Others were only found in only one of the samples. Consequently transient superinfections may be frequent even in the healthy population. Persisting strains were not found in all samples between their first and last detection, probably due to fluctuations of the viral load caused by superinfections with other TTV variants [Dencs et al., 2009].

Detection and genotyping of swine TT viruses in different organs of weaned piglets from a Hungarian piggery

Prevalence of swine TTV (TTSuV) variants was measured in serum samples of 44 weaned piglets. Sixteen liver samples and 22 intestinal samples were also tested. PCR products obtained from three organs of one piglet were sequenced and a phylogenetic analysis was performed.

TTV viraemia was common among the piglets (32/44, 72.7%), but several of the liver and intestinal samples were also positive (5/16 and 4/22, respectively). In case of one piglet all three samples gave positive results, and a more detailed analysis was carried out. The nucleotide sequences obtained from the serum ad the intestine of the piglet were very similar. The liver however contained a viral genome that was only distantly related to the sequences found in the other two organs and to the TTSuV isolates previously published in GenBank. Based on recently published sequence data and classification system, the virus detected in the serum and the intestinal samples belong to TTSuV genotype 1b. The sequence obtained from the liver represents genotype 1c. Thus this was a case of a mixed infection, where different TTV genotypes were present in different organs of the host, probably because of the different

tissue specificities of the variants. This phenomenon has already been published for human TT viruses [Takács et al., 2008].

In the course of our work concerning recently discovered human hepatitis viruses and the related swine TT viruses we determined their prevalence in different populations and collected data on their most prevalent genotypes in Hungary.

Molecular investigation of hepatitis B virus outbreaks

We analysed the genetic relatedness of the hepatitis B viruses detected in the serum samples of two children and their foster mother, to find if there was a connection between the adoption and the infection of the foster mother. The children had been infected perinatally by their natural mother and became virus carriers despite active and passive immunization.

By sequencing a segment from the S region of the genome of the detected viruses we found that all three viruses belonged to subgenotype D1 and their nucleotide sequence was identical in the analysed region. This suggested that the source of the virus infecting the foster mother was the virus of one of the children [Szomor et al., 2002].

In case of the other outbreak, serum samples of 7 children treated at the onco-haematology unit of a hospital in Hungary and also the serum of one of the children's brother were tested for the presence of HBV. Nucleotide sequence of PCR products were determined and a phylogenetic analysis was performed. A nosocomial outbreak was suspected after several family members of one of the patients were diagnosed with acute HBV infections. Hepatitis B virus DNA could be detected in all 8 HbsAg children. A segment of the S region of the genome was sequenced.

The analysis of the obtained HBV sequences showed that the viruses all belonged to genotype D. They were also very closely related, which suggested the infections were from a common source. They also showed great homology with sequences from another nosocomial HBV outbreak, which occurred several years earlier at another hospital. The onco-haematology departments of the two hospitals had been collaborating for years and some of the patients waiting for the bone marrow transplantation attended both units. Our analysis provided molecular evidence for the relatedness of the two outbreaks. This connection probably would have been missed by a conventional epidemiological investigation. The on-site inspection revealed several deficiencies in infection control practices, which together may have led to the occurrence of the nosocomial outbreak.

Molecular investigation of nosocomial hepatitis C outbreaks

Molecular epidemiological investigations were carried out in case of two suspected health care associated hepatitis C virus outbreaks. One of them was reported from a haemodialysis center, the other from the oncology unit of a hospital.

Several patients of a small satellite dialysis unit were temporarily moved to the haemodialysis center for treatment because of technical problems at the smaller unit. Two months later anti-HCV seroconversion was observed in case of 17 patients of the two units. Eleven of the seroconverted patients were shown to be HCV RNA positive. The viruses of 4 known HCV carriers treated at the center were also included in the investigation. Nucleotide sequencing and phylogenetic analysis of the NS5B region was performed.

The sequence analysis showed that the viruses of the 11 newly infected patients were genetically closely related, suggesting the infections had a common origin. The viral sequences obtained from 3 of the known HCV carriers appeared to be independent from the outbreak. Their evolutionary distance from the other patients' viruses (and from each other) were similar to those of international reference sequences. The HCV sequence obtained from the fourth known virus carrier (P12) was genetically very close to the sequences from the outbreak. HCV nucleotide sequences from the 11 newly infected patients and from patient P12 were placed on a common branch on the phylogenetic tree. This clustering of the sequences was also confirmed by the bootstrap analysis. Our results suggest that a nosocomial outbreak of hepatitis C virus infections occurred at the haemodialysis center and that the common source of infections was HCV carrier patient P12 [Dencs et al., 2009b]

Twenty patients, treated at the the oncology department of a hospital, with no previous record of hepatitis C infection, showed elevated liver enzyme levels and tested positive for anti-HCV antibodies in 2008. The common origin of the infections was assumed, so a molecular epidemiological investigation of the possible nosocomial outbreak was performed. Another patient had been a known virus carrier for years, and was considered a possible common source of infections. Serum samples of 13 patients were positive for HCV RNA, including the sample from the long time carrier patient. First the NS5B region, then the more variable E1/E2 region of the genome was analysed.

Sequence analysis showed that *de novo* infected patients were infected with subtype 1a. They formed two groups on both phylogenetic trees, but the groups were closely related. This confirmed that they were involved in a health care associated HCV outbreak. Bootstrap analysis also confirmed the relatedness of the sequences from the newly infected patients. The virus infecting the long time HCV carrier was shown by genotyping to belong to subtype 1b,

thus it could be excluded as the source of the outbreak. The common source of the infections could not be identified in case of this outbreak. The infections may have originated from an HCV carrier patient who was not identified by the field investigation. This patient probably carried a highly divergent virus population. Different quasispecies were selectively transmitted to and became dominant in the exposed patients, whose viral sequences consequently formed two groups in the phylogenetic analysis.

The precise mode of transmission could not be ascertained neither in the haemodialysis center, nor in the oncology ward. After the introduction of strict infection control measures no more infections were identified in either of the institutions.

Viruses of 40 HCV carrier control patients unrelated to the outbreak were genotyped to identify the most common HCV genotypes in Hungary. Genotype 1 proved to be predominant (38 patients). Only a single subtype 1a virus was found, all others belonged to subtype 1b. The viruses of the two other patients were classified into genotypes 3 and 4.

In summary, during our investigations concerning hepatitis B and C viruses we successfully applied molecular epidemiological techniques to confirm or to exclude the common origin of the infections. The genome segments amplified and sequenced were shown to be long and variable enough not only to genotype and subtype the detected viruses, but also to determine their genetic relatedness with the help of controls and reference sequences. Should another outbreak occur, our methods can quickly provide molecular data, which may help in preventing the further spread of the virus [Dencs et al., 2011].

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