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Research paper

Genomic changes detected after serial passages in cell culture of virulent human G1P[8] rotaviruses

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ABSTRACT

Serial passages of a virulent mouse rotavirus in cell cultures caused a loss of virulence in mice. To gain insight into the genomic mutations in human rotavirus during cell culture and its attenuation in humans, we serially passaged three wild type human G1P[8] rotavirus strains (Wa, DC3695, DC5685) derived from diarrheal stool samples up to 60 times in two different cell cultures (human colon adenocarcinoma cell line: HT29, and primary African green monkey kidney cells: primary AGMK). We sequenced the whole genomes of 60 times-passaged strains and compared them with those of the original viruses. Most substitutions were detected in VP4, followed by substitutions in VP7 and NSP4 genes. Substitution at amino acid 385 in the putative VP4 fusion domain and substitution T45M in NSP4 genes were detected in all AGMK-passaged strains, respectively. These genomic changes are likely to correlate with a loss of rotavirus virulence in humans.

1. Introduction

Rotaviruses (RVs) form a genus of the *Reoviridae* family and are divided into at least eight groups (species), designated A–H/I (Matthijnssens et al., 2012; Mihalov-Kovás et al., 2015). Among these, group A RVs are one of the most important etiologic agents of severe diarrhea in children, causing approximately 453,000 deaths each year among children <5 years of age in the world, predominantly in developing countries (Tate et al., 2012).

The RV genome consists of 11 segments of double-stranded RNA and encodes six structural proteins (VP1–4, 6, and 7) and six nonstructural proteins (NSP1–6) (Estes and Greenberg, 2013). Traditionally, the 2 outer capsid proteins (VP7 and VP4) independently induce neutralizing antibodies and are the basis of a dual classification system defined by G and P types, respectively. Recently, RV genotyping classification system based on all 11 genes was proposed to analyze RV virulence and evolution in detail (Matthijnssens et al., 2008). This classification system demonstrated that most human RV strains have been classified into three groups, that is two major groups: Wa-like (G1/3/4/9-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) and DS-1-like (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2), and one minor group: AU-1-like (G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3).

In a study based on a semi homologous system of gnotobiotic piglets infected with a virulent porcine RV, an attenuated human RV, or their reassortants, Hoshino et al. (1995) revealed that (i) VP3, VP4, VP7 and NSP4 genes of porcine RV each play an important role in the virulence of RV infection in piglets; and (ii) all four of these porcine RV genes are required for the induction of diarrhea and the shedding of porcine/human reassortant RVs in piglets. These observations provide a potency of new strategy for the attenuation of wild-type human RV, from the point of developing a safe and effective vaccine.

It is well known that the serial passage of a virulent wild-type virus *in vitro* often lose virulence in the original animal host, whereas the serial passage of a cell-culture-adapted avirulent virus *in vivo* often results in a reversion to virulence in the original animal host (Hanley, 2011). In a previous study, we alternately and repeatedly serially passaged a murine RV in mouse pups and in cell culture, and showed that at least three genes encoding VP4, NSP1, and NSP4, are involved in the pathogenicity of murine RVs in mice (Tsugawa et al., 2014). The phenomenon of viral attenuation during serial passages in cell culture has been widely used to produce live-attenuated viral vaccines. One of the currently available attenuated RV vaccines, *i.e.*, Rotarix® (GlaxoSmithKline, GSK), was developed using this strategy. However, the mechanisms underlying this attenuation have not been fully clarified.

In this study, we serially passaged three wild-type human G1P[8] RV strains derived from diarrheal stools in two different cell culture systems: HT29 cells (a human colon adenocarcinoma cell line; homologous cell culture) and primary African green monkey kidney cells (primary

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AGMK; heterologous cell culture). We then sequenced the whole genome of 60 times-passaged strains and compared them with those of each original strain to identify the common amino acid changes to all three strains that could be responsible for a possible attenuation of the viruses.

2. Materials and methods

2.1. Viruses

The three human G1P[8] RV strains used in this study were derived from symptomatic hospitalized children with diarrhea in the USA. The Wa strain was isolated in 1974 (Wyatt et al., 1980). The two DC strains, DC3695 and DC5685, were isolated in 1989 and 1991, respectively. All stool samples were stored at −80 °C until used.

2.2. Cell cultures and serial passages

Two cell culture systems, the human colon adenocarcinoma cell line HT29 and primary African green monkey kidney cells: primary AGMK (Diagnostic Hybrids, Athens, OH), were used in this study. The viruses were activated at 37 °C for 30 min with 5 μg/ml trypsin (Sigma γ–irradiated trypsin), and adsorbed on to the cells at 30 °C for 1 h under 5% CO_2. The first passage was performed with 10% stool suspensions in roller tube cultures at 37 °C with 0.5 μg/ml trypsin. At the time of 50% of the infected cells displayed cytopathic effects, the cultures were harvested. Then, the cell lysates were passaged (i) undiluted or diluted 10^{-1}–10^{-3} before the 20th passage and (ii) diluted arbitrarily 10^{-1}–10^{-8} after 20th passage to avert genome rearrangement (Hundley et al., 1985). In this way, the viruses were serially passaged 60 times in roller tube cultures of HT29 cells or primary AGMK cells.

2.3. RNA extraction, RT–PCR, and nucleotide sequencing

Virus RNA was extracted with TRIzol Reagent (Invitrogen) from 10% stool suspensions and culture lysates. We performed RT–PCR and nucleotide sequencing for original stool samples and 20, 40 and 60 times-passaged samples in 2 cell cultures, as described previously (Tsugawa and Hoshino, 2008; Tsugawa et al., 2014). The primers used in this study are listed in Supplementary Table 1.

2.4. Determination of the 5′ and 3′ terminal viral gene sequences

The complete nucleotide sequences of all 11 segments, including the 5′ and 3′ terminal sequences, of the original stool samples and 40 times-passaged samples were performed by a full-length amplification of cDNAs (FLAC) method, as described previously (Maan et al., 2007; Tsugawa and Hoshino, 2008; Tsugawa et al., 2014). The specific primers for FLAC are also listed in Supplementary Table 1.

2.5. Sequence analysis

The sequences were analyzed with Sequencher 5.2 (Gene Codes Corporation). In the case of virus samples composed of mixed populations possessing original or mutated sequences, the dominant virus was determined by signal intensity of chromatogram. If there were no difference between them, we regarded original and mutated in parallel.

The GenBank/EMBL/DDBJ accession numbers for the sequence of the original stool samples and the 20, 40, and 60 times-passaged samples used in this study have been submitted to the GenBank nucleotide sequence database under accession numbers in Supplementary Table 2.

3. Results

3.1. Summary of genotype constellations of three RV strains from human stools

The complete genotype constellations and nucleotide sequence identities of the original three G1P[8] RV strains from human stools used in this study were compared with those of the Wa strain, as shown in Supplementary Table 3. All three strains were classified as having a Wa-like genotype constellations (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1).

3.2. Mutations detected after serial passages in HT29 and AGMK cell cultures

All gene mutations and amino acid substitutions detected in each of the 11 genes after 20, 40 and 60 times passaged in HT29 and AGMK cell cultures are summarized in Supplementary Table 4a–c. The total number of gene mutations and amino acid substitutions increased with the gain of passages. Table 1 summarized the numbers of amino acid substitutions detected after 60 passages in two cell cultures. Totally, more than five substitutions passaged in each cell cultures were detected in VP4, VP7 and NSP4 genes. These three genes also had high nonsynonymous substitution rate (63 to 100%) in each cell cultures. Next, we searched common amino acid substitution to all three strains, which had been passaged 60 times. We found out only one common substitution in each VP4, VP7 and NSP4 genes; that is D385N or D385H of VP4 in AGMK cell-passaged, Q201R of VP7 in HT29 cell-passaged, and T45A of NSP4 in AGMK cell-passaged strains. These amino acid substitutions and gene mutations were showed with their state in 20 and 40 times-passaged strains (Table 2).

3.3. Change of 3′ CS of NSP1 gene after serial passages in cell cultures

The 3′ consensus sequence (3′CS; UGU GACC) was conserved after serial passages in all 11 segments with the exception of NSP1 gene. In NSP1 gene, although the GACC motif at the 3′-end was conserved in all stool samples, it was lost in 4 of 6 strains serially passaged 40 times in cell culture, *i.e.*, it was altered to GGCC in HT29-cell-passaged Wa and DC3695 strains, and in AGMK-cell-passaged DC5685 strain; and to GAACC in AGMK-cell-passaged Wa strain.

4. Discussion

Point mutations and reassortment play important roles in RV evolution and vaccine development. The currently available RV vaccines in the world, Rotarix® (live-attenuated vaccine) and RotaTeq® (reassortant vaccine) were developed using the point mutation and reassortment mechanisms, respectively. Although the efficacy of these RV vaccines has been established, the mechanism of their attenuation has not been fully clarified (Ruiz-Palacios et al., 2006; Vesikari et al., 2006).

It is important to confirm the attenuation of virulent RV strain with *in vivo* study for the development of safe vaccines. However, it was ethically difficult to perform *in vivo* human study to confirm the attenuation of RV strains. Previously, we revealed that virulent murine RV EB strain was attenuated in mice after serial 18 times passages (Tsugawa et al., 2014). Yuan et al. (1996) demonstrated that human G1P[8] RV Wa strain was attenuated in gnotobiotic pigs after 6 consecutive times limiting dilution cloning and further 27 times passages in cell cultures. At the development process of G1P[8] RV live-attenuated vaccine (Rotarix®), this vaccine candidate passaged serially 33 times in cell cultures was identified to be almost attenuated to humans with the exception of minor adverse effect (mild fever) in clinical trial (Bernstein et al., 1999). Then, after further 3 consecutive times limiting dilution cloning and 7 times passages (total serially 43 times passages) in cell cultures,