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Development of an *in vitro* cell culture model for human noroviruses and its clinical application

Key Messages

1. Despite the encouraging results on murine norovirus 1, there is no evidence for the successful cultivation of human norovirus (hNoV) in cell lines of haematopoietic lineage. Further effort to develop an *in vitro* cell culture system for hNoV is needed.
2. Using *ex vivo* cultures, we found that hNoV displays a previously unreported marked tropism to human duodenal glandular epithelial cells. Future research to evaluate the permissiveness of human cell lines of this type for hNoV infection is warranted.

Introduction

Human norovirus (hNoV), a member of the family *Caliciviridae* in the genus *Norovirus*, is the leading cause of acute non-bacterial gastroenteritis worldwide, and affects all age groups in both developed and developing countries. An overwhelming majority of sporadic cases as well as outbreaks were attributed to strains classified into genogroup II genotype 4 (GII.4). In the past decades, research on the development of a culture system or a small animal model for hNoV replication has made little progress. The failure of culturing hNoV has severely hampered the study of the pathogenesis of this important enteric pathogen and corresponding antiviral strategies. Attempts to cultivate hNoV using a broad panel of cell lines have been unsuccessful.[1] It has been reported that hNoV can infect differentiated human embryonic small intestinal cell line (INT 407) when grown as a three-dimensional culture,[2] but whether productive viral replication occurs remains unclear.[3]

Murine norovirus 1 (MNV-1) has recently been demonstrated to infect and replicate in murine macrophage cell lines and cultured primary dendritic cells and macrophages from STAT1$^{-/-}$ mice.[4] This unexpected virus tropism to cells of haematopoietic lineage has provided new insights into the possible development of an *in vitro* culture system for hNoV. We therefore set out to establish an *in vitro* cell culture model for hNoV based on the hypothesis that human and murine NoVs share similar tropism to cells of haematopoietic lineage. We summarise our efforts to cultivate hNoV in these cell types *in vitro* and detail our exploration of alternative approaches.

Study design and methods

This study was conducted from January 2006 to December 2007. We investigated the permissiveness of five haematopoietic cell lines (KG-1, THP-1, J774A.1, RAW264.7, and WBC264-9C) of human and murine origin for a panel of diverse hNoV strains that covered two genogroups and seven genotypes. We assessed successful viral infection and productive RNA replication by light microscopy and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) targeting the junction between ORF1 and ORF2 of the viral genome. We also evaluated whether *STAT1* mRNA silencing makes the cell lines permissive for or better able to support hNoV replication. Furthermore, we evaluated whether *ex vivo* culture of human adult duodenal tissues can be used for the study of hNoV. We assessed successful viral infection and productive replication using RT-qPCR, *in situ* hybridisation, and immunohistochemical staining.

Results

Non-permissiveness of different cell lines of haematopoietic lineage for hNoV

Apparent CPE was observed in cell lines RAW264.7 and WBC264-9C when compared with mock inoculated cells and with cells inoculated with heat-inactivated virus (data not shown). However, only cell enlargement (a morphological change) was noted (data not shown). There was no evidence of cell lysis or foci formation. Using RT-qPCR, in all the cell lines tested, we

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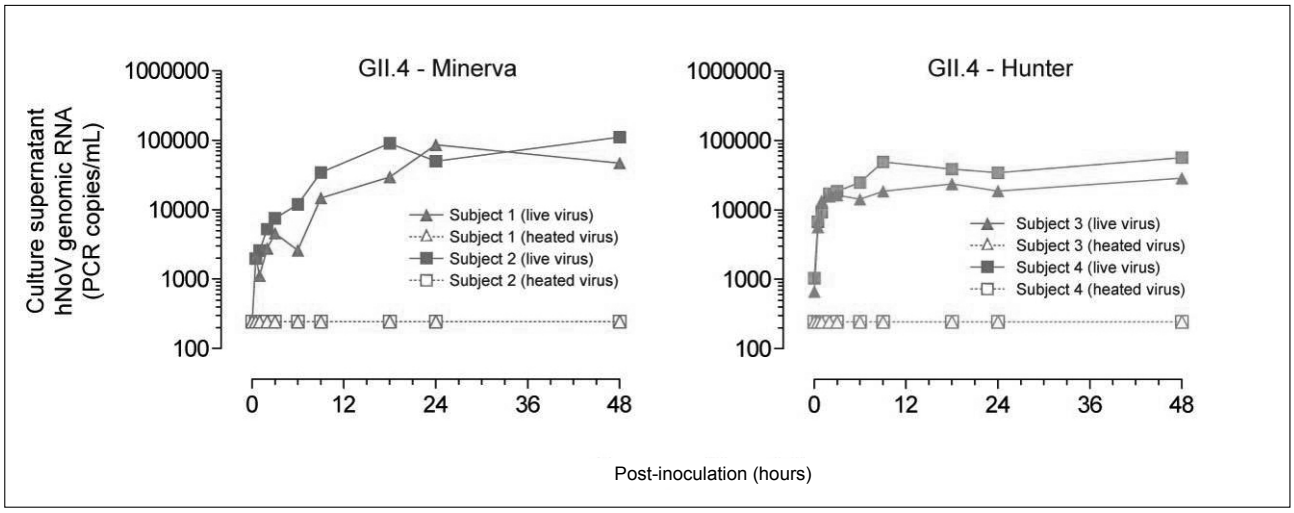

Fig 1. RNA growth kinetics of human norovirus (hNoV) in *ex vivo*–inoculated adult human duodenal tissues
Viral RNA growth curves of two recent epidemic GII.4 strains (Minerva and Hunter) are shown. Viral genomic RNA levels in cell-free culture supernatants are measured by RT-qPCR targeting the ORF1/2 junction

observed no substantial increase (>5 fold) in viral genomic RNA level up to 7 days post-inoculation (data not shown). Moreover, plaque assays were all negative (data not shown). Similar results were observed in all the five cell lines when STAT1 mRNA was silenced even after achieving substantial reductions of the mRNA and protein levels of STAT1, as confirmed by RT-qPCR and Western blotting up to 3 days after siRNA silencing (data not shown). Collectively, our data did not support the proposition that the five haematopoietic cell lines investigated were permissive for hNoV.

Infection and productive replication of hNoV in ex vivo culture using adult human duodenal tissues

We explored an alternative approach to study hNoV replication. We evaluated whether *ex vivo* culture can be used for the study of hNoV by inoculating freshly collected human duodenal tissues obtained from adult dyspeptic volunteers using two recent epidemic hNoV GII.4 strains (Minerva and Hunter). Aliquots of *ex vivo* culture supernatant were collected at 0, 0.5, 1, 2, 3, 6, 9, 18, 24, and 48 hours post-inoculation and clarified by centrifugation. We found that viral genomic RNA levels in cell-free culture supernatants increased over time as measured by RTqPCR (Fig 1).

To determine whether the productive hNoV RNA replication in *ex vivo* culture was accompanied by viral proteins expression, we performed immunohistochemical staining against structural VP1 as well as non-structural viral material. We detected intracellular cytoplasmic expressions of both VP1 (Fig 2a) and viral protease (Fig 2b) 48 hours post-inoculation. Detection of newly synthesised viral protease further supports the possibility that viral replication was occurring *ex vivo*.

We then performed *in situ* hybridisation to detect and locate intracellular hNoV RNA on tissues that were formalin-fixed and paraffin-embedded 48 hours post-inoculation. Digoxigenin-labelled antisense RNA probe that hybridises to and detects positive-stranded viral genomic RNA by targeting the ORF1/2 junction gave positive signals in both the cytoplasm and nuclei of infected cells (Fig 3a). This corroborates our RT-qPCR findings that hNoV can infect human duodenal tissues in *ex vivo* culture. Notably, positive signals were observed predominantly in glandular epithelial cells and were less frequently noted in cells on luminal epithelial surfaces (Fig 3a). The results showed that hNoV had a marked tropism for glandular epithelial cells of the human duodenum. This cell tropism was confirmed by comparable immunohistochemical staining patterns of structural VP1, and non-structural viral protease produced by *in situ* hybridisation (Fig 2). We also observed positive staining using the sense RNA probe that hybridises to and detects negative-stranded viral antigenomic RNA (Fig 3b). This indicates that hNoV was actively replicating in *ex vivo* cultures, through the production of negative-stranded RNA.

Limited viral RNA replication in a glandular epithelial cell line

We then selected and investigated the permissiveness of a glandular epithelial cell line known as human intestinal epithelial cell line 6 (HIEC-6) for a GII.4 strain of hNoV (Hunter) as described above. Limited productive RNA replication as evident by an up to 2-$\log_{10}$ increase in viral genomic RNA level in cell-free culture supernatant was detected 48 hours post-inoculation (data not shown). However, no observable CPE was noted in this cell line.

Discussion

The absence of a culture system for hNoV propagation and the non-permissiveness of replicon-based reverse genetics systems for this infection have severely hampered study of

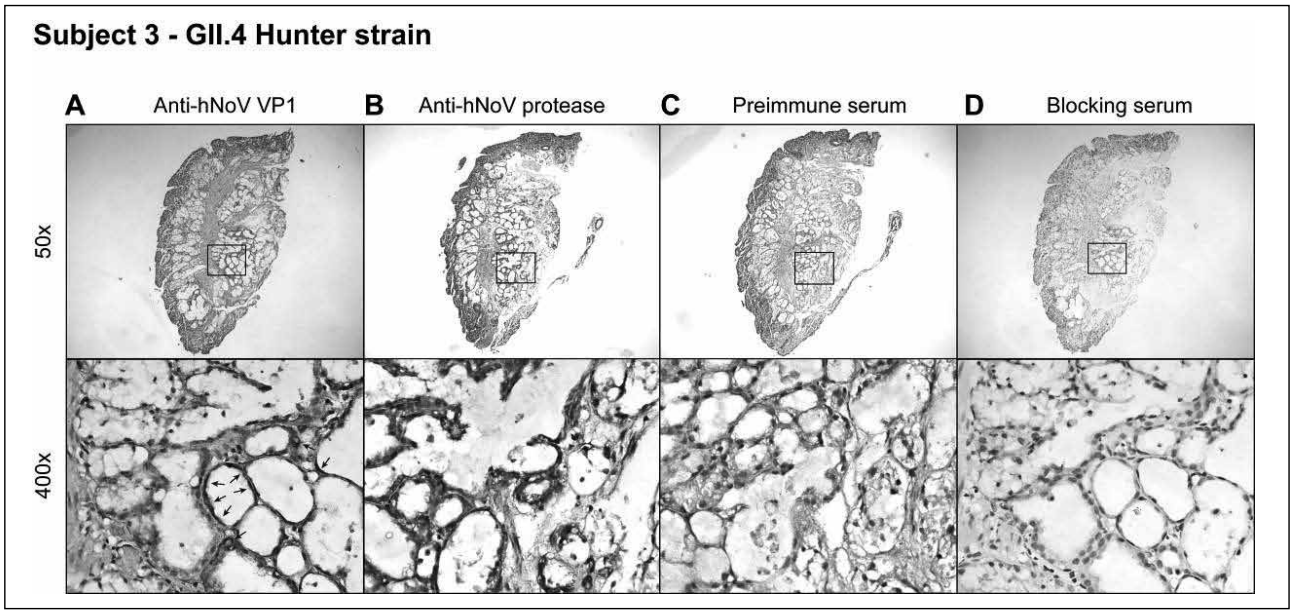

Fig 2. Immunoperoxidase staining for human norovirus (hNoV) structural VP1 and newly synthesised non-structural viral protease in *ex vivo*–inoculated adult human duodenal tissues

(a and b) Formalin-fixed paraffin-embedded sections (48 hours post-inoculation) are stained with either mouse monoclonal anti-hNoV VP1 antibody, (a) NS14 or (b) rabbit polyclonal anti-hNoV protease antiserum, followed by DAB colour development. Positive signals (dark patches or dots) for VP1 (arrows) and viral protease are observed in the cytoplasm of infected cells resembling glandular epithelial cells. Sections are counterstained with Harris haematoxylin. (c) Preimmune serum of anti-hNoV protease antiserum and (d) blocking goat serum in replace of primary antibody are used as antibody specificity controls. Sections from volunteer subject 3 infected with Hunter strain are shown

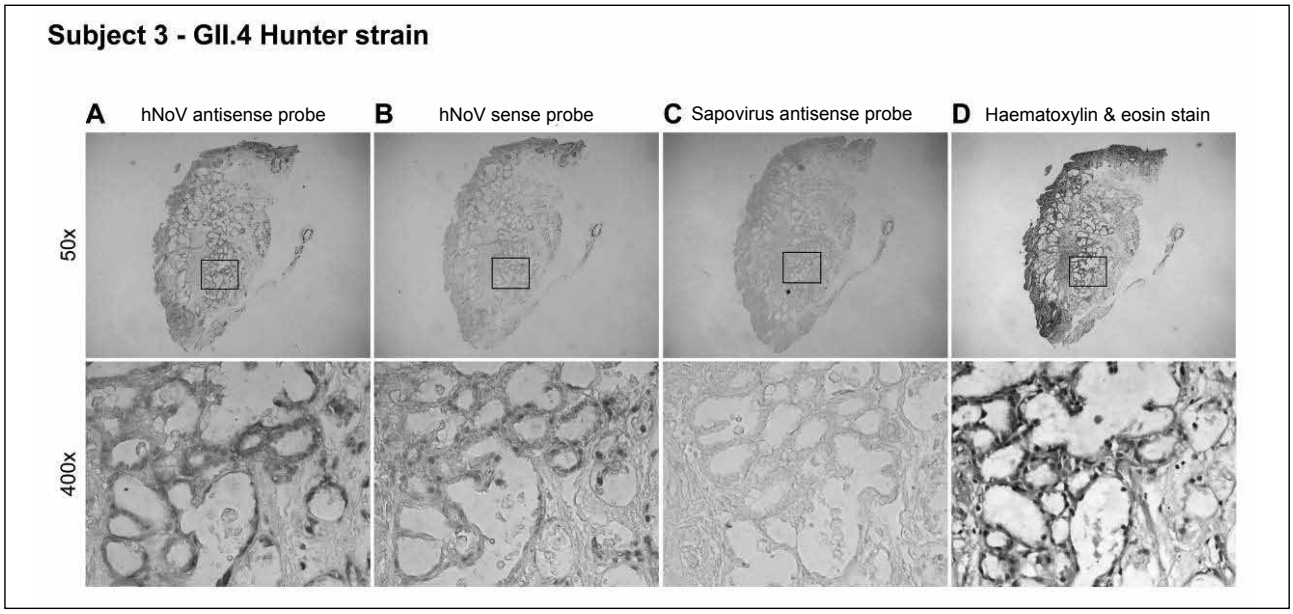

Fig 3. *In situ* hybridisation for human norovirus (hNoV) genomic and antigenomic RNA in *ex vivo*–inoculated adult human duodenal tissues

(a and b) Formalin-fixed paraffin-embedded sections (48 hours post-inoculation) are hybridised with (a) digoxigenin-labelled antisense or (b) sense hNoV RNA probe targeting the ORF1/2 junction, followed by NBT/BCIP colour development. Positive signals (dark patches or dots) are noted in both the cytoplasm and nuclei of infected cells resembling glandular epithelial cells using antisense probe that hybridises to and detects (a) positive-stranded viral genomic RNA but are only noted in nuclei of infected cells using sense probe that hybridises to and detects (b) negative-stranded viral antigenomic RNA. (c) Sections are hybridised with unrelated digoxigenin-labelled antisense RNA probe targeting capsid gene sequence of human sapovirus. No observable signals are detected, confirming the positive signals in panels A and B are hNoV-specific. (d) Sections are stained with Harris haematoxylin and eosin

its pathogenesis and putative antiviral strategies.

In this study, five cell lines of haematopoietic lineage were investigated, after they were selected based on previous work that showed that MNV-1 can infect and replicate in them.[4] We hypothesised that human and murine NoVs share similar tropism to cells of haematopoietic lineage. Although apparent CPE was observed in cell lines RAW264.7 and WBC264-9C, no productive RNA replication was detected using RT-qPCR. Subsequent investigation of the apparent CPE showed no plaque formation. Since haematopoietic cells isolated from STAT1$^{-/-}$ mice had been shown to better support MNV-1 replication,[4] we evaluated whether silencing *STAT1* mRNA can render the cell lines permissive for hNoV. However, findings observed were similar to those with their non-silenced counterparts. We cannot exclude the possibility that siRNA treatment itself may pre-activate STAT1-independent anti-viral interferon response that in turn may inhibit hNoV replication in these silenced cell lines. The non-permissiveness of their non-silenced counterparts have been shown by other groups to readily support murine NoV replication, which casts serious doubts in regard to further investigation of these haematopoietic cell lines for hNoV cultivation. Collectively, our data do not support the notion that haematopoietic cell lines can support hNoV replication *in vitro*.

We then explored the use of an alternative approach to study hNoV. *Ex vivo* culture using freshly collected human tissues usually mimics the primary site of viral replication *in vivo*. This method has been successfully applied to studies of infection and replication of a diverse range of clinically important viruses such as the highly pathogenic avian influenza (H5N1) virus.[5] In this regard, we performed *ex vivo* culture of two recent epidemic GII.4 strains using freshly collected adult human duodenal tissues obtained from adult dyspeptic volunteers. Our findings illustrate that *ex vivo* culture can support key stages of complete hNoV replication, ranging from virus adsorption and internalisation to viral RNA replication and protein synthesis. Notably, *in situ* hybridisation of viral RNA and immunohistochemical staining of VP1 and newly synthesised viral protease revealed that hNoV displays a marked tropism for glandular epithelial cells. This previously unreported cell tropism suggests that human cell lines derived from or resembling intestinal glandular epithelial cells may support hNoV replication and merit investigation.

In this regard, we selected the glandular epithelial cell line HIEC-6 based on our ex vivo culture findings and evaluated its permissiveness for hNoV. HIEC-6 was human intestinal epithelial cells isolated from foetal ileal tissue. This was shown to express intestinal crypt cell marker antigen MIM-1/39.[6] Although CPE was not observed upon hNoV inoculation, limited viral RNA replication of a hNoV GII.4 strain (Hunter) was noted in this cell line. This suggests that cell lines of similar types are promising candidates worthy of further investigation. We are screening other glandular epithelial cell lines for their ability to support hNoV replication and, more importantly induce cytopathic effects that are crucial to the development of a plaque assay to quantify viable virus.

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