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Temperature and density dependent induction of a cytopathic effect following infection with non-cytopathic HAV strains

Samantha Q. Wales, Diana Ngo, Kaoru Hida, Michael Kulka *

Division of Molecular Biology, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 8301 Muirkirk Road, Laurel, MD 20708, USA

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ABSTRACT

Hepatitis A virus infection and growth in cultured cells is protracted, cell-type restricted, and generally not accompanied by the appearance of a cytopathic effect, with the exception of some culture-adapted strains. We demonstrate that the non-cytopathic HAV strain HM175/clone 1 can be induced to exhibit a cytopathic phenotype in both persistently or acutely infected cells under co-dependent conditions of lower incubation temperature ($<34\ ^{\circ}C$) and reduced cell density in both monkey (FRhK-4) and human (A549) cells. This phenotype is not virus-strain restricted, as it was also observed in cells infected with HAV strains, HAS-15 and LSH/S. Cytopathic effect was accompanied by rRNA cleavage, indicating activation of the RNase L pathway, viral negative strand synthesis, caspase-3 activation, and apoptosis. The results indicate that a cytopathic phenotype may be present in some HAV strains that can be induced under appropriate conditions, suggesting the potential for development of a plaque assay for this virus.

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Introduction

Hepatitis A virus (HAV) is a member of the picornavirus family of RNA viruses and currently the only member of the genus Hepatovirus (Norder et al., 2011). This virus remains the major causative agent of infectious hepatitis worldwide and is transmitted primarily via the fecal-oral route; its dissemination via person-to-person contact as well as contaminated food and water has been documented and is the subject of numerous reviews (Cliver, 2010; Fiore, 2004; Koopmans and Duizer, 2004). HAV is stable, relatively resistant to inactivation and decontamination methods often successful against non-enteric viruses, and can remain infectious under varying environmental conditions as well as on environmental surfaces for substantial periods of time.

Early research investigations achieved the ability to culture HAV derived either directly from human samples or subsequent to passage of human isolates in monkeys. For example, Provost and Hilleman (1979) demonstrated that primary cell cultures of monkey origin were permissive for HAV replication and supported the production of infectious virus (human isolate) post-passage in monkeys. Frösner et al. (1979) reported the use of an established cell culture line that was permissive to replication of HAV directly following virus extraction from an outbreak (human fecal) sample. Other investigators (Flehmig, 1980, 1981; Flehmig et al., 1981) studied the direct application of human stool samples containing HAV to a rapidly growing, established cell line without the requirement for additional agents such as the presence of HBV genome in the cell line used by Frösner et al. (1979). Subsequent work from these as well as other investigators revealed significant results regarding the culture adaptation of wild-type strains (Hollinger, 2001; Nainan et al., 2006; Ross et al., 1991). For example, HAV infection and growth in cultured cells is cell-type restricted; permissive replication is typically demonstrated in primate cells of non-human origin. However, the human cell line A549 and the embryonic diploid cells WI-38 and MRC-5 have also been shown to be permissive for at least some strains of HAV.

Following acute infection in culture, HAV replication is slow, requiring weeks/months for consistent detection of expressed viral antigen and/or particles without onset of a cytopathic effect and thus can establish a persistent (steady-state) infection. Serial passage/sub-culture of HAV strains can result in culture-adaptation of the virus typically defined by increased virus replication rates and expression of viral antigen, and has been achieved with various strains from human isolates as well as monkey passaged human virus (Binn et al., 1984; Bradley et al., 1984; Daemer et al., 1981; Flehmig, 1980; Flehmig et al., 1981; Frösner et al., 1979; Gauss-Müller et al., 1981; Kojima et al., 1981; Provost and Hilleman, 1979). Indeed, these persistently infected cell lines can be routinely sub-cultured, as with the uninfected parental line, often for up to a year without overt induction of apoptosis, cpe or dramatic changes in morphologic appearance, but with

* Corresponding author. Fax: +1 301 210 7976.
E-mail addresses: samantha.wales@fda.hhs.gov (S.Q. Wales), diana.ngo@fda.hhs.gov (D. Ngo), kaoru.hida@fda.hhs.gov (K. Hida), michael.kulka@fda.hhs.gov (M. Kulka).

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viral RNA, protein, and infectious virus recovery from these cells. The reason(s) for the protracted replication rate of wild-type virus and culture-adapted strains (despite the improved replication of the latter compared to the former) is not completely known; however, it has been posited that HAV, which lacks a host shut-off function typical of other picornaviruses, competes for critical cellular factors essential for virus gene expression and replication (Bedard and Semler, 2004; Pinto et al., 2007). Alternatively, it has been suggested that slow replication and virus particle maturation/yield may be the consequence of ongoing encapsidation of the viral RNA pool, asynchronous replication, down regulation of viral RNA replication or rate-limiting viral translation (Anderson et al., 1988; Cho and Ehrenfeld, 1991; De Chastonay and Siegl, 1987; Funkhouser et al., 1999).

The serial passage of cell-culture adapted non-cytopathic HAV strains has resulted in the emergence of even faster growing strains capable of inducing a cytopathic effect (cpe) during virus replication. Interestingly, the induction of cpe appears to be restricted to particular cell lines, for example FRhK-4 (and their derivative Frp/3), B-SC-1 and A549 cells (Ali, 2002; Anderson, 1987; Cromeans et al., 1989; Cromeans et al., 1987; Divizia et al., 1986; Gaspar et al., 1992; Lemon et al., 1991; Nasser and Metcalf, 1987; Venuti et al., 1985); the reason(s) for this cell restricted phenotype is not yet fully understood. In this regard, both non-cpe and cpe HAV strains remain of interest to investigators particularly those studying the processes responsible for viral replication and cpe induction by HAV and the contribution of mutations in the viral genome to these processes (Beneduce et al., 1995; Brack et al., 1998; Cohen et al., 1989; Emerson et al., 1991; Funkhouser et al., 1994; Jansen et al., 1988; Lemon et al., 1991). Induction of cpe is also of interest to investigators in providing a mechanism by which to quantitate viral replication. Given the non-cytopathic nature of wild-type and culture-adapted HAV strains, early investigators applied techniques such as radioimmunoassay (RIA), direct and indirect immunofluorescence, and immune electron microscopy to evaluate whether virus replication and gene expression occurred in HAV infected cells. The quantification/titration of HAV infectivity later included established methods such as radioimmunofocus assays (RIFA), immunofluorescence, ELISA, RT-PCR, immunological-based focus assays, and in situ hybridization (Jansen et al., 1985; Lemon et al., 1983; Richards and Watson, 2001; Siegl et al., 1984a; Yeh et al., 2008). Notably, a plaque assay has not been developed for either wild-type or culture-adapted HAV due to the absence of cytopathogenicity of these strains. However, there is a real need for a relatively simple quantitative methodology that does not involve radionuclide(s) and demonstrates both infectivity and production of infectious progeny, all features that a plaque assay would provide.

During the present investigation, we discovered that culture incubation temperature can affect the phenotype of a culture-adapted, non-cytopathic HAV strain (HM175/clone 1) during persistent infection. We then sought to determine what other incubation and infection parameters can affect the regulation and expression of this phenotype. Our results indicate that (i) culture incubation temperature in combination with cell culture density affect the temporal onset of cpe, (ii) cpe is the result of the induction of caspase-dependent apoptosis in persistently infected cells and is commensurate with caspase activation in acutely infected cells, (iii) development of cpe is not the result of an abortive infection as viral protein (capsid) and negative strand synthesis is readily detected during infection, and (iv) the induction of cpe occurs during acute infection with three culture-adapted, non-cytopathic HAV strains and, therefore, is not solely a viral strain restricted phenomenon. Interestingly, the induction of cpe/apoptosis in infected cells is preceded by rRNA cleavage (viz. activation of the OAS/RNase L pathway) and therefore may be dependent on the activation of the OAS protein. We postulate that other non-cytopathic HAV strains may also be similarly affected to induce apoptosis/cpe in a manner that is likely co-dependent on these and other factors such as virus replication rate, the presence/absence of critical cell regulatory factors and possibly the activation of the RNase L pathway.

Results

Induction of cpe phenotype in HM175/clone 1 infected cells during incubation at 33 °C

In our laboratory, the regular growth and maintenance of FRhK-4 (monkey) cells, as well as FRhK-4 cells persistently infected with HAV HM175/clone 1 (also called clone 1 cells (Kulka et al., 2009)) are typically conducted at an incubation temperature of 37 °C (Goswami et al., 2004; Kulka et al., 2009; Kulka et al., 2003). Clone 1 cells can be sub-cultured on a weekly basis up to 240 days post infection (dpi) without the appearance of cpe and preliminary studies in our laboratory have shown that continuous sub-culture for up to approximately 1 year pi is possible, albeit with subtle morphologic changes becoming increasingly visually evident as the period of sub-culture increases (beginning at approximately 250 days pi) (data not shown). During routine sub-culturing we discovered that incubation of persistently infected cells at 34 °C resulted in the onset of a "cpe-like" morphology after 7 days (data not shown). Continued investigation revealed that the induction of cpe was visually discernible beginning at approximately 3–4 days growth at either 33 °C or 34 °C (data not shown). As discussed in the Materials and Methods, we conducted further investigations at 33 °C unless otherwise stated. In Fig. 1, the effect of 33 °C incubation is shown for FRhK-4 cells either uninfected or persistently infected with HM175/clone 1 using brightfield microscopy (panels (A)–(C)). Persistently infected cells cultured at 37 °C (panel (B)) have a morphologic appearance similar to that observed for uninfected FRhK-4 cells cultured either at 37 °C (data not shown, Goswami et al., 2004) or 33 °C (panel (A)). When persistently infected cells are cultured at 33 °C, the development of cpe-like morphology is observed beginning at day 4 (data not shown). Both the number and the severity of cells exhibiting this morphology increases with increasing time of incubation at 33 °C and is shown for persistently infected cells at day 6 (panel (C)), and it is easily distinguishable from similarly sub-cultured cells incubated at 37 °C (panel (B)).

We were interested in determining whether the apparent temperature-dependent morphologic change observed in the infected FRhK-4 cells is restricted to a given cell-type. To this end we established an HM175/clone 1 persistently infected A549 (human) cell line as these cells have been reported previously to support the replication of HAV (Cromeans et al., 1989). As shown in Fig. 1, persistently infected A549 cells cultured at 37 °C (panel (E)) demonstrate little morphologic differences from uninfected A549 cells at either 37 °C (data not shown) or 33 °C (panel (D)). As with FRhK-4 cells, cpe-like morphology develops at approximately 4 days (data not shown) and increases with time of incubation at 33 °C and is readily apparent at day 6 (panel (F)), and it appears that both persistently infected cell lines grow more slowly at 33 °C than at 37 °C (data not shown). Importantly, the results indicate that induction of cpe is co-dependent on HAV infection and incubation temperature, and is not cell type restricted. Indeed, we believe that the induction of cpe during cell culture infection with a non-cytopathic strain of HAV is a significant discovery since as far as we are aware it has not been previously reported in the literature.