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Human respiratory syncytial virus reduces the number of cells in S-phase and increases GADD153 expression in HEp-2 cells

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Summary. – Human respiratory syncytial virus (HRSV) associated with bronchiolitis and asthma is known to replicate actively in ciliated epithelial cells. However, little is known about the influence of HRSV replication on the cell cycle. We found that HRSV infection of HEp-2 cells led to a reduction of the number of cells in S-phase, to an increase in the number of cells in G1-phase, together with an increase of GADD153 mRNA levels and GADD153 protein expression. These results implied that a shorter S-phase supported HRSV replication suggesting possible strategies for interfering with productive HRSV infection.

Keywords: Human respiratory syncytial virus; cell cycle; S-phase; G1-phase; GADD153

HRSV (the genus *Pneumovirus*, the family *Paramyxoviridae*) is the most important cause of lower respiratory tract infection in infants and young children worldwide and a risk factor for the development of asthma (Lukacs *et al*., 2007; Hansbro *et al*., 2008). In the US alone, HRSV causes 4 million cases of respiratory tract infection annually, resulting in about 125,000 hospitalizations (Meissner, 1994; Shay *et al*., 1999, 2001). Efforts to develop effective vaccines for HRSV have met with failures (Castilow *et al*., 2007; Power, 2008). Identifying a mechanism underlying host-virus interactions can help to design useful clinical strategies.

One of the most important aspects of virus-host interactions is the effect of virus replication on the regulation of cell cycle. Viral effects on the cell cycle can determine whether the infected cells enter apoptosis or become tumorigenic or whether the infecting virus enters a latent stage. Very little is known about the impact of HRSV infection on the cell cycle. Therefore, we determined the percentage of cells in different stages of the cell cycle after infection (Table 1). HRSV infection led to a decrease of the number of cells in S-phase with an accompanying increase of the number of cells in the G1-phase. The impact of HRSV infection on the cell cycle may explain an unresolved issue that HRSV infection does not lead to an increase of apoptosis despite activation of genes associated with the apoptosis (Bitko *et al*., 2007).

To determine the impact of HRSV infection on the cell cycle, HEp-2 cells were infected at a multiplicity of infection (MOI) of 1 or 5 in 1 ml of media. After 1 hr of adsorption at room temperature, 10 ml of fresh medium was added to the plates. HEp-2 cells were able to support a productive viral infection with CPE detectable 48 hrs post infection (p.i.). Twenty-four hours p.i., the infected cells were harvested and resuspended in 200 µl of PBS. The cells were fixed with 2 ml of 70% ethanol in PBS and later stained with 500 µl of 40 µg/ml propidium iodide and 40 µg/ml RNaseA (Sigma). The stained cells were analyzed by flow cytometry. The cells

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Abbreviations: F protein = fusion protein; G protein = attachment protein; GADD153 = G1 arrest, DNA damage-153; HRSV = Human respiratory syncytial virus; IL = interleukin; MOI = multiplicity of infection; p.i. = post infection

Table 1. HRSV infection reduces the number of cells in S-phase

Exp. No.	Cell cycle phase	% of mock-infected cells	% of HRSV-infected cells (MOI = 5)
1	G1	49	65
	S	18	7
	G2/M	33	28
2	G1	51	61
	S	23	6
	G2/M	26	33
3	G1	52	59
	S	24	9
	G2/M	24	33

infected with HRSV at MOI = 1 had a modest change in the number of cells in S-phase, but the cells infected at MOI = 5 showed significantly fewer cells in S-phase compared to the mock-infected cells (Table 1). HRSV infection also showed an increase in the number of cells in the G1-phase. At 24 hrs p.i., the number of cells in the HRSV-infected sample was reduced by 40% compared to the mock-infected cells (data not shown).

The preparation of infecting virus contains IL-1β that has been shown as an inhibitor of the transition from G1- to S-phase in some cell types. However, available methods for virus purification do not sufficiently remove non-viral components, i.e., do not remove completely non-viral soluble factors. Therefore, we employed two strategies to confirm the involvement of HRSV infection in our experiments. First, we treated the purified viral preparations with 100 μg/ml of anti-F protein antibody against envelope F glycoprotein that neutralizes virus infectivity. Treatment with anti-F protein antibody abolished HRSV-mediated reduction of the number of cells in S-phase (Table 2). These results were consistent with those of Schlender *et al*. (2002) for HRSV infection of lymphocytes. However, anti-G protein antibody directed against envelope G glycoprotein had only slight, if any, effect on the HRSV-mediated reduction of the number of cells in S-phase.

To further substantiate the conclusion that HRSV infection and not a component of the infecting virus was responsible for the cell cycle effects, we utilized RhoA 77-95 peptide that is known to block the viral infectivity by interacting with the F protein using a mechanism similar to that of other polyanion inhibitors (Budge *et al*., 2004a,b). Treatment of the preparation of HRSV with 200 μg of the RhoA 77-95 peptide at the time of infection completely inhibited the effect of the reduction of S-phase cells. In contrast, the control peptide, RhoA 87-105, had no effect (Table 3; Fig. 1). These data suggested that the reduction of the cell number in S-phase was specific for the virus infection and that F protein might be involved in mediating of this effect.

Inhibition of the HRSV-mediated effect by the anti-F protein antibody strongly suggested that this effect was due to the initial stages of viral infection, perhaps at the initial interaction of the virus with the cell. Cells simultaneously incubated with the RhoA 77-95 peptide and HRSV followed by extensive washing with PBS before addition of fresh media, did not manifest a reduction in the number of cells in S-phase (Table 4). However, the HRSV-infected cells incubated with RhoA 77-95 peptide after virus infection and

Table 2. Anti-F protein antibody inhibits HRSV-mediated reduction of the number of cells in S-phase

Exp. No.	Cell cycle phase	% of mock-infected cells	% of HRSV-infected cells	% of HRSV + anti-F protein antibody-infected cells	% of HRSV + control antibody*-infected cells
1	G1	49	65	49	59
	S	18	7	23	18
	G2/M	33	28	28	29
2	G1	50	61	50	65
	S	27	16	32	17
	G2/M	22	23	18	18

*anti-G protein antibody in exp. 1 and irrelevant antibody in exp. 2.

Table 3. RhoA 77-95 peptide inhibits the HRSV-mediated reduction of cell number in S-phase

Exp. No	Cell cycle phase	% of mock-infected cells	% of HRSV-infected cells	% of HRSV + RhoA 77-95-infected*	% of HRSV + control peptide-infected cells*
1	G1	46	61	46	61
	S	21	7	22	8
	G2/M	34	32	22	31
2	G1	–	–	50	63
	S	–	–	23	9
	G2/M	–	–	27	29

*Peptide RhoA 77-95 or control peptide Rho 87-105 were added simultaneously with the virus. (–) = not done.

following PBS wash, did manifest the reduction in S-phase cells caused by infection. These data support the likelihood that virus attachment or early infection events led to the inhibition of G1- to S-phase transition.

To determine a possible mechanism for the reduction of the number of cells in S-phase, we conducted a DNA microarray analysis. The preliminary results indicated a virus mediated induction of GADD153 mRNA, which was consistent with a reduction of the cell number in S-phase. This finding implied that GADD153 expression led to the G1 arrest. Real-time PCR analysis confirmed the preliminary results from the microarray experiment (Fig. 2). To conduct real-time PCR analyses, the following primers were used: GADD153 forward = 5'-AAACGGAAACAGAGTGGT CATTC-3'; GADD153 reverse = 5'-TGCTTGAGCCGT TCATTC-3'; β-actin forward = 5'-ATTGCCGACAGGAT GCAGAA-3'; β-actin reverse = 5'-GCTGATCCACATCT GCTGGAA-3'.

The expression of GADD153 protein was also assayed by a Western blot analysis using anti-GADD153 antibody F-168 (Santa Cruz). Twenty four hrs p.i., we observed a 50% increase in GADD153 protein expression in the HRSV-infected cells (Fig. 3).

The above data were consistent with the conclusion that HRSV infection blocked the cells in G1-phase, although there was no explanation for the lack of proportionality between the considerable raise in the GADD153 mRNA level versus the modest increase in GADD153 protein expression following HRSV infection. Previous reports indicated that HRSV activated the expression of STAT1α, interferon regulatory factor-1 and interleukin-1β-converting enzyme, all of which facilitate apoptosis (Young *et al*., 2000; Liu *et al*., 2004). However, HRSV infection does not lead to the apoptosis. In general, there are two strategies for prevention of the apoptosis, which is associated with a deregulated entry into S-phase in the absence of appropriate growth stimuli. The first strategy is employed by many DNA tumor viruses and facilitates growth factor independent activation of a mitogenic signaling pathway. The second strategy regulates

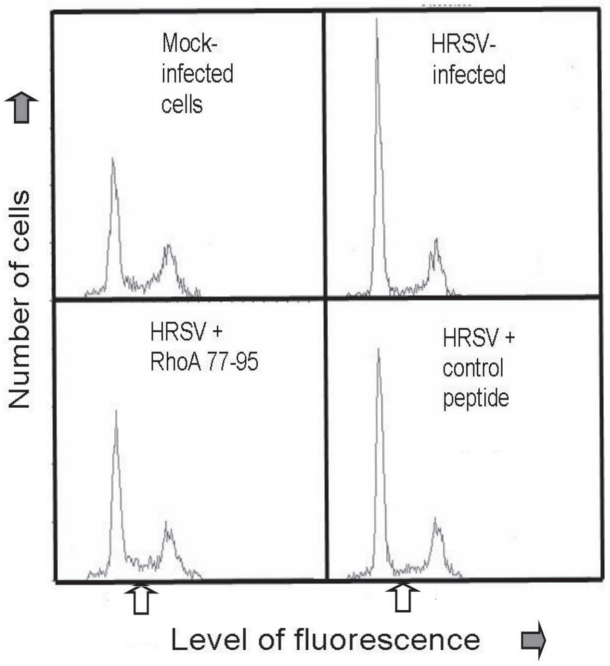

Fig. 1

Flow cytometry of HRSV-infected cells treated with RhoA 77-95 peptide

Open arrows indicate the cells in S-phase. RhoA 87-105 peptide was used as a control.

entry of cells into S-phase by a stimulation of retinoblastoma protein expression. Data above indicated that a lack of the apoptosis in HRSV-infected cells was attributable to the second strategy, possibly via the increased expression of GADD153 protein. However, GADD153 protein is induced as a part of cell death program that occurs when the unfolded protein levels are too high. GADD153 expression reduces bcl-2 levels and NF-κB activation leading to the apoptosis (Kim *et al*., 2006). Thus, a possible role of GADD153 protein in HRSV infection remains to be determined.

Table 4. RhoA 77-95 peptide inhibits the HRSV-mediated reduction of the cell number in S-phase in the early stage of infection

Exp. No	Cell cycle phase	% of mock-infected cells	% of HRSV-infected cells	% of HRSV + RhoA 77-95*	% of HRSV + RhoA 77-95**
1	G1	50	60	46	57
	S	23	10	23	12
	G2/M	27	30	31	30
2	G1	–	–	52	60
	S	–	–	26	11
	G2/M	–	–	23	30

*The peptide was added together with the virus. **The peptide was added after virus adsorption. (–) = not done.

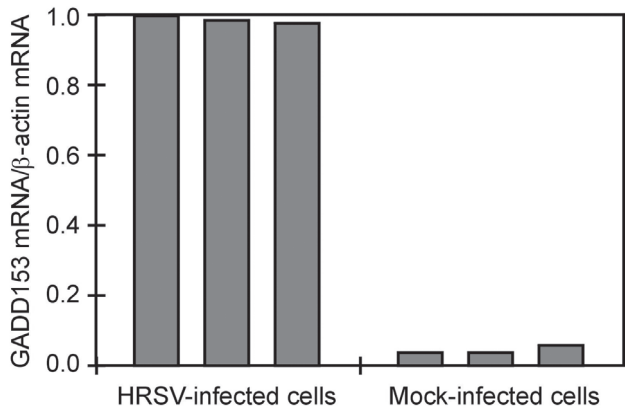

Fig. 2

Real-time PCR assay of GADD153 mRNA in HRSV-infected cells

Data shown represent three independent experiments.

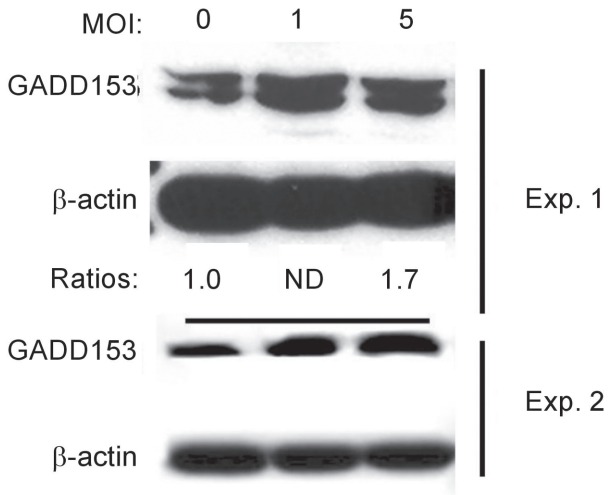

Fig. 3

Western blot analysis of GADD153 protein in HRSV-infected cells

Ratio refers to level of GADD153 normalized to β-actin.

Taken together, we addressed the basic mechanism by which HRSV infection affected the cell cycle. This effect could by blocked by antibodies or peptides that interacted with F protein. This finding suggested that early events of HRSV infection as the initial virus-cell interaction or the virus entry resulted in the inhibition of G1- to S-phase transition.

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