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Nuclear phenotype modifications in human cell cultures after infection with encephalomyocarditis virus

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ABSTRACT

RD human rhabdomyosarcoma cells and HEK 293-human embryonic kidney cells were infected with 0.1 TCD_{50}/cell of the Columbia-SK strain of encephalomyocarditis virus (EMCV). EMCV was propagated in the RD and HEK 293 cell line and induced apoptosis. Resistant cells of the both cultures which surviving after lytic infection had differentiated phenotypic modifications, and can characterized by suppressed malignancy(suppressed proliferation rate, decreased DNA amount, increased euploidy, decreased of the average number of nucleoli).

INTRODUCTION

Encephalomyocarditis virus (EMCV) is a rodent picornavirus belonging to the *Cardiovirus* genus and has an extremely wide range of hosts including humans.

The RD cell line, established by McAllister et al.[20] from a human rhabdomyosarcoma, supported the replication of some picornaviruses, including coxsakie viruses and poliovirus. Our studies were conducted to further explore the sensitivity of the RD cells for propagation of the EMCV. HEK 293 cells supported the growth of some picornaviruses-coxsackievirus and echovirus[4]. HEK 293 cells were generated by transformation of human embryonic kidney cell cultures (hence HEK) with sheared adenovirus 5 DNA, and were first described in 1977[8]. Our studies were conducted to further explore the sensitivity of the RD and HEK 293 cells for propagation of the EMCV and investigate some characteristic responses associated with viral cytopathogenesis.

MATERIALS AND METHODS

Virus

EMCV (Columbia-SK strain) was obtained from the Institute of Virology, NSA of Russia, Moscow and was used at multiplicity of infection 0.1 TCD_{50} per cell 48h after reseeding. Infected and intact cells were incubated at 37^0C. Viral titres in TCD_{50}/ml were calculated by the method of Kärber. The virus titres in medium and cells were investigated at 6, 12, 24, and 48 h post infection.

Cells

RD, a continuous rhabdomyosarcoma culture, was cultured in Eagle's medium with 10% bovine serum with an additional 2mM L-glutamine and 1mM sodium pyruvate. A monolayer of intact cells was used 48h after

passage. Cells were reseeded by a single 10^5 cell/ml dose. Cell viability was measured by trypan blue staining.

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's MEM containing 10% fetal bovine serum with an additional 2mM L-glutamine and 1mM sodium pyruvate. A monolayer of intact cells was used 48 h after passage. Cells were reseeded by a single 10^5 cell/ml dose. Cell viability was measured by trypan blue staining.

Image cytometry

Cells were fixed in 96% ethanol for 30min and treated with fresh Schiff's reagent for Feulgen staining (hydrolysis in 5N HCl, 60min at 22°C). Nuclear and nucleolar DNA content was determined using a computer-equipped microscope-photometer SMP 05(OPTON). The television method was used at 575nm. In each group of cells with different numbers of nucleoli, 50-100 cells were measured. The total number of cells investigated at each incubation time was about 500; i.e. about 2000 cells altogether. Nuclear DNA content, area and perimeter were simultaneously determined. In the same nuclei, we defined the outlines of nucleoli by the limits of perinucleolar and intranucleolar chromatin, then determined the DNA content, area and perimeter of each nucleolus. Each of these nucleolar indices was summed over all the nucleoli in each nucleus to give total nucleolar values and nucleolar/nuclear ratios of area, perimeter and DNA content. DNA content was expressed as a "c" scale, where 1 "c" is half (haploid) the nuclear DNA content of cells from a normal (non-pathological) diploid population in G_0/G_1 phase. Unstimulated lymphocytes were used as standards.

Apoptosis detection

DNA loss and changes in chromatin texture in apoptotic RD cells were evaluated by image analysis of Feulgen-stained preparations[14,17].

RESULTS

Viral propagation in cell line

To preliminarily assess viral cytotoxicity and apoptosis induction, RD and HEK cells were infected with the EMCV virus at a multiplicity of infection of 0.1 TCD_{50} per cell. Infected RD cells arrested cell growth for 12h p.i., involved cytopathic effect, and reduced the cell number to less than 10% of that of non-infected cells at 24h p.i. The peak of viral titer was at 24h p.i.(3.5 $\log_{10}$). Infected HEK cells arrested cell growth for 12h p.i., involved cytopathic effect, and reduced the cell number to 28% of that of non-infected cells at 24h p.i. The peaks of viral titers were at 24-48 h p.i.(4.0 $\log_{10}$).

Infected cells were observed for appearance of the viral cytopathic effect. The virus was titrated in RD and HEK cells. EMCV titers in RD and HEK cells were lower than those in HEp-2 (7.5 $\log_{10}$). EMCV replicated in RD cells with very high speed. At 12 h after infection cells were phenotypically changed, and after 24h p.i. most of cells in the culture (84.6±9.1%) were dead. By 36 h there was no monolayer to measure. Cytopathic effects were observed after 12 h p.i. The cells appeared to separate and become rounded, with eventual detachment and formation of defects in the cell monolayer. The cytopathic action of the virus led to massive cell death, with surviving cells (after 24h p.i.) numbering less than 10% of non-infected cells. Survived cells had phenotypic modification such as small size, decreased DNA amount in nuclei, and decreased average number of nucleoli in population (figures 1, 2, 5, 6). EMCV replicated in HEK cells with high speed. At 12h after infection cells were phenotypically changed,

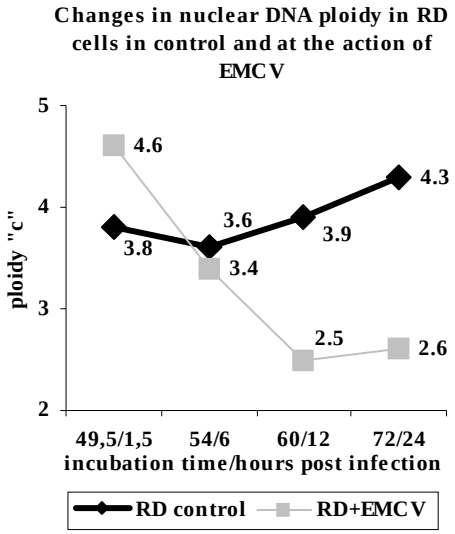

Figure 1: Changes in nuclear DNA ploidy in RD cells in control and at the action of EMCV

Regular Paper

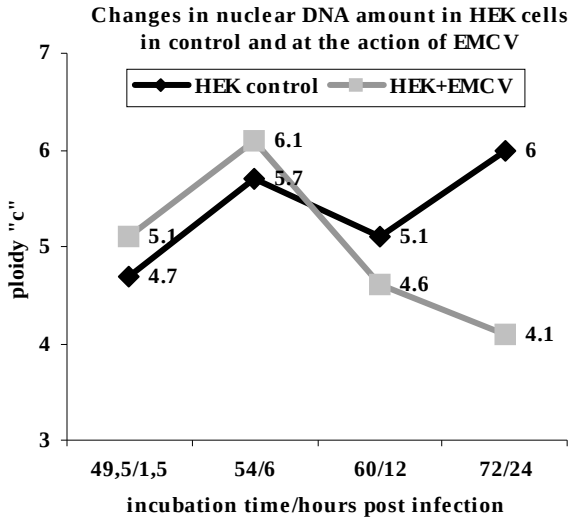

Figure 2: Changes in nuclear DNA amount in HEK cells in control and at the action of EMCV

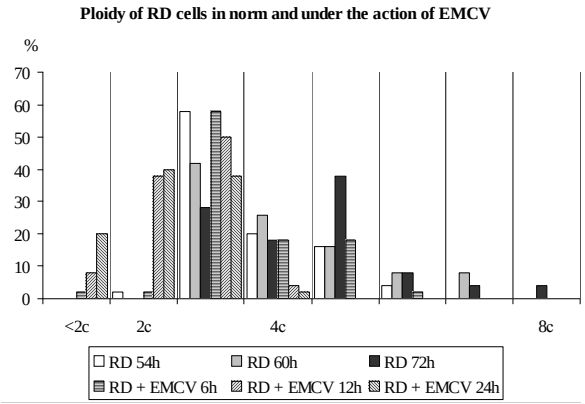

Figure 3 : Distribution of RD cells by the ploidy for control and EMCV infected RD cells (%)

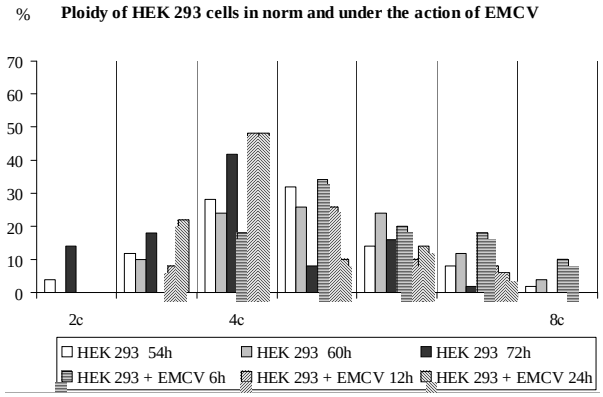

Figure 4 : Distribution of HEK 293 cells by the ploidy for control and EMCV infected HEK 293 cells (%)

and after 24h p.i. most of cells in the culture(71.8±4.2%) were dead. By 48 h there was no monolayer to

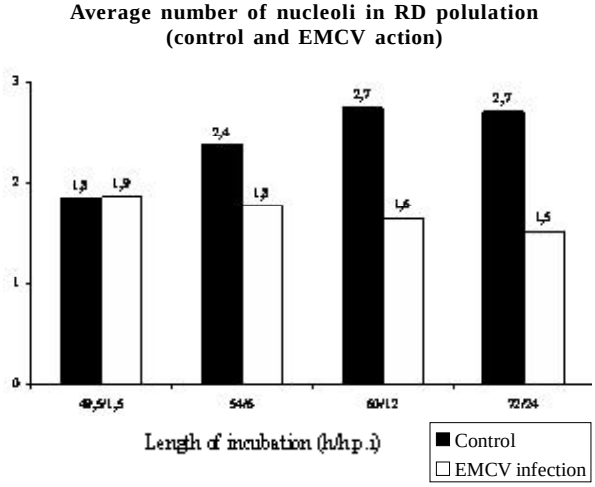

Figure 5 : Changes in number of the nucleoli per nuclei in RD control and under the EMCV action

TABLE 1 : Apoptosis/mitosis ratio for control and EMCV infected HEK and RD cells

Groups	Length of incubation h./h. p.i.					
	54/6		60/12		72/24	
	Control	EMCV infection	Control	EMCV infection	Control	EMCV infection
HEK	0.8	1.1	2.68	1.26	1.38	1002.9**
RD	2.0	0.89	2.65	5.33*	1.0	126.3**

***Significant compared to control, p<0.05, **Significant compared to all incubations and controls, p<0.01-p<0.001**

measure.

Nuclear and nucleolar indices

Intact RD cell line was hypotetraploid (average ploidy of the summarized passages was 3.88 "c"±0.17). Here, under the action of the EMCV the ploidy began to decrease by 12h after infection and at 12 and 24h the difference between infected and control cells had become significant (2.58"c"±0.23, p<0.001) (figure 1).

Intact HEK cell line was hypertetraploid (average ploidy of the summarized passages was 5.38 "c"±0.41). Here, under the action of the EMCV the ploidy began to decrease after infection and at 24h the difference between infected and control cells had become significant (4.09"c"±0.33; p<0.01) (figure 2).

The average number of nucleoli per nucleus in control RD population increased with incubation time (TABLE 1). Comparing the 49.5h with the 60 and 72h populations, an increase is apparent in the relative numbers of, 4 and 5 nucleolar cells and there is a corresponding decrease in 1 and 2 nucleolar cells. Under the EMCV infection in RD cells, the number of nucleoli

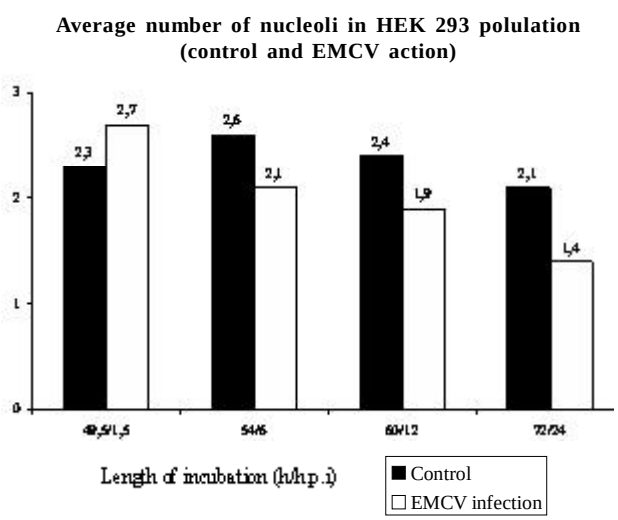

Figure 6 : Changes in number of the nucleoli per nuclei in HEK 293 control and under the EMCV action

was markedly reduced beginning 6h p.i. (figure 5). Cells with four and more nucleoli per nucleus completely disappeared and there were few with three nucleoli. Most of the cells of the population (more than 90%) had a single or two nucleoli.

Intact HEK 293 cells gave different results (figure 6). The average numbers of nucleoli per nucleus were stabile in all control incubations. But under the EMCV infection in HEK cells, like in RD cells, the number of nucleoli was markedly reduced beginning 24h p.i. Multinucleolar cells completely disappeared, and most of the cells of the population also had a single or two nucleoli.

In summarized passages of intact RD cells 23.0±1.18% of the interphase cells were euploid. As follow from the figure 3 the percentage of euploid and nearly euploid cells in the infected RD culture increased at a significant rate. Most of euploid cells in the controls (more than 80% of the total) were 4 "c" and 8 "c" and few diploid cells were present, but EMCV infection generated a dominant population of diploid cells (more than 90% of euploid population). Under the action of the EMCV the percentage of euploid and nearly euploid cells in the infected culture significantly increased - 20.4% control 72h, 36.0% 24h p.i. EMCV($p<0.05$). In summarized passages of intact HEK cells 26.5 ±3.77% of the interphase cells were euploid (figure 4). Like RD cells under the action of the EMCV the percentage of euploid and nearly euploid HEK cells in the infected culture significantly increased -42% at 12h p.i. and 24h p.i. ($p<0.05$-$p<0.01$ to corresponding controls).

So our studies in revealed numerous phenotypic alterations in cells, of susceptible lines resistant to viral infection. We showed that these cells displayed decreased nuclear size, decreased DNA amount, and decreased number of nucleoli per nuclei.

Viral cytotoxicity and apoptosis induction to RD and HEK cells

Changes in chromatin texture in RD and HEK cells were evaluated by image analysis of Feulgen-stained preparations. Most part of RD cells in 12 and 24h p.i. show classic characteristics of apoptosis[14]. The same preparations were used to quantify apoptotic and mitotic indices (AI/MI) and the ratio of these parameters (TABLE 1). As follow from the figure 3, cells with apoptotic phenotype when the nuclei showed chromatin condensation typical of apoptosis (with clumps of condensed chromatin distributed at the nuclear periphery, or larger areas of condensed chromatin and a reduced nuclear area[14] were observed in significantly increased quantities beginning 12h p.i. This growth continued up to 24h p.i., after which most cells in cultures were dead and only single cells survived (less then 20%).

DISCUSSION

This is a first report of an adaptation of EMCV to propagation in HEK cell line. Replication of EMCV in RD cells has been reported in 1983[21].

Cell death by apoptosis is now widely considered to be a host response to limit virus replication through elimination of virus-infected cells[13]. During viral replication (including EMCV replication) produced double-stranded RNA(dsRNA), which triggers a rapid apoptosis in the susceptible cells. Efficient apoptosis in response to viral dsRNA results from the cooperation of the two major apical caspases(8 and 9) and the dsRNA-activated protein kinase R/ribonuclease L system that is essential for the inhibition of protein synthesis in response to viral infection[11]. DsRNA inhibited protein synthesis by phosphorylation of the eukaryotic translation initiation factor eIF2α on serine-51 by PKR[9]. Individual expression of human IFN-mediators has shown that overexpression of 2'5' A syn-

Regular Paper

thetase[3] or p68 kinase[19] confers resistance to EMCV.

Although the presence or absence of virus receptors on the cell surface remains a major determining factor of the susceptibility of a cell to virus infection, were shown that the intracellular environment plays an important role in the result of viral infection. Our data show that only cells with specific nuclear phenotype were more resistant to EMCV infection. RD and HEK cells that survived 24 h after EMCV infection showed lower DNA ploidy and increased euploid population. One of the mechanisms that could explain our data is regulation of the cell cycle by the picornaviruses[5]. EMCV can deblock cells localized in the G_2 phase. This can explain also changes in the structure of euploid population (significantly increased total euploid cells and population of diploid cells in RD culture).

Another plausible explanation of these changes can be action of interferon-induced proteins, like P69. P69 expression caused inhibition of replication of encephalomyocarditis virus but not of vesicular stomatitis virus, Sendai virus, or reovirus. There was increasing accumulations of the P69-expressing cells in the G_1 phase of the cell cycle[7].

The average number of nucleoli decreased significantly in the HEK 293 and RD populations in the EMCV-infected group. In both cultures the average number of nucleoli per nucleus changed markedly after infection, and the population distribution of this parameter also changed. Cells with more than 4 nucleoli per nucleus disappeared completely and there were few with three nucleoli. Most of the cells in both populations (more than 50%) became single-nucleolar; only about 36% had two nucleoli. The number of nucleolar-forming regions is genotypically determined[6,22], so differences among subgroups of multinucleolar cells are more likely than the production of new clones. Multi nucleolar cells are probably more labile to the viral infection than those with 1 or 2 nucleoli. This phenomenon can be explained as selective cytotoxicity of EMCV in RD and HEK populations, and one of possible mechanisms of decreasing of cellular concentration of rRNA during EMCV infection, relative to control samples[1].

Interestingly, a resistant cells which surviving cells after lytic infection had differentiated phenotypic modifications, and can characterized by suppressed malignancy (suppressed proliferation rate, decreased DNA amount, increased euploidy, decreased of the average number of nucleoli). The attachment of EMCV to human cells susceptible to virus infection is mediated by a cell surface VCAM-1 or VCAM-1 like receptor[12,10]. Another picornaviral receptors (like CD155 for poliovirus), selectively expressed on a wide variety of tumor cells[2,15,18]. So selective cytotoxicity might be a result of overexpression of the EMCV receptors on the multinucleolar cells. Selective cytotoxicity might also be related to the fact that certain EMCV replication processes depend on the cell cycle[23]. Also, the chosen viral dose gave possibility that some cells will surely survive the first viral cycle (6-10 hrs to lysis), and up-regulate interferon or other antiviral signals that work to protect adjacent cells from subsequent infection and it also can be result that the cells of RD and HEK lines, resistant to lytic infection with the EMCV virus, show a changed phenotype.

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