

Peptides of the Hinge Region of Ezrin Suppress Replication of Hepatitis C Virus in Human Cells *In Vitro*

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Adaptation of cells to external conditions is provided by special signalling systems which connect the cell membrane with cytosol and nucleus. The human protein ezrin is very important in the co-ordination of cellular responses to extracellular signals. Ezrin connects adhesion molecules with the cytoskeleton and with the PI 3 Kinase signaling pathways, inositol-3-phosphate and diacylglycerin. Through various pathways, ezrin actively takes part in the processes of cell polarization and locomotion, the formation of the cell to cell and cell to extracellular matrix contacts, in cell protection mechanisms from viral infection⁹⁻¹³. Ezrin is present in cell in two conformations: active and inactive. Active form of ezrin is associated with cell membrane, while the inactive form is located in the cytosol. The transition from the inactive to active form proceeds as a result of conformational change in hinge region of ezrin, which is located between amino acids 330 to 350 from N- end of protein molecule⁹.

We have already reported that synthetic peptides that duplicate fragments of hinge region of ezrin have immuno-modulating and antiviral activity. Among other things, it was discovered that under influence of peptides from the hinge region of ezrin there was an amplification of immune reactivity to different antigens⁶. It was also shown that ezrin peptides 324-337, 333-346 and 343-355 suppressed the development of encephalomyocarditis virus in an infected culture of human cells⁷. These hinge region peptides of ezrin protected laboratory animals from lethal infection, that was induced by Herpes Simplex Virus type I⁸. This particular paper describes antiviral action of the synthetic peptides identical to part of the hinge region ezrin against hepatitis C Virus (HCV) in human cell culture.

Materials and Methods

Peptides

Peptides that mimic parts of the hinge region of human ezrin^{14,15} particularly TEKKRRETVEREKE (amino acids 324 to 337 from N-terminus of the ezrin polypeptide chain), EREKEQMMREKEEL (333 to 346) and KEELMLRLQDYEE (343 to 355) were synthesised by S. Pomoguybo.

Virus

We used the cytopathogenic variant of Hepatitis C Virus (HCV), strain C-13 related to genotype1b⁴. This strain was isolated from blood serum taken from a patient suffering from chronic viral hepatitis C, identified as hepatitis C virus in a neutralisation assay with antibodies specific for HCV and also in reactions of haemagglutination, immuno-enzyme analysis, immuno-fluorescence and diffusion

precipitation in agar. RNA of the virus was identified as HCV genome in RT-PCR by using primers to 5'-NTR and HCV region which codes for the nucleocapsid protein, and also by sequencing of genome of the HCV fragment that codes for nucleocapsid protein region of HCV²⁻⁴.

The cells used to culture HCV were of SW-13 human cells from an adrenal gland adenocarcinoma and highly sensitive to cytopathogenic effect. (the culture has been deposited in the American National collection of cell cultures). Cell culture SW-13 was grown in Igla double medium with 10% serum of Fetal Calf Serum, with addition of glutamine and antibiotics (100Unit/ml).

HCV was inoculated on a one-day monolayer of SW-13 cells, which were grown in 24-well plastic micro-titre plates. Cells culture SW-13 was infected with HCV in dose 10 TCD₅₀/ml. The infective activity of HCV was assessed by the results of titration on Day 6 after infection, when the maximum cytopathogenic effect was developing.

Research of antiviral effects of drugs.

Peptides 324-337, 333-346 and 343-355 in concentrations 0.1, 1 and 10 mcg/ml were added to the SW-13 cell cultures at the moment of infection and 24 hours after the cell cultures were infected by HCV. The control drug used was Reapheron (NPO "Vector" Novosibirsk), a well known for its antiviral activity to HCV infection in cell culture. Reapheron was used at a concentration of 5000 UN/ml. The assessment of the drug's antiviral effect was based on reduction or reverse of the cytopathogenic effect of HCV, and also on the HCV titre reduction in liquid cell cultures of SW-13, infected with HCV at a dose of 10 TCD₅₀/ml.

Titration of HCV in liquid culture.

Samples of culture supernatants were taken on Day 3 after cells were infected. These samples were used for 10 serial dilutions for infection of bulk cell cultures, sensitive to HCV. In 48-well plastic plates a serial dilution of samples of 200 microlitres was made. After that, 500 microlitres of cell suspension on the double growth medium was added, which contain 4% of Fetal Calf Serum. After incubation at room temperature for 20 minutes the infected cultures were placed in a thermostat incubator for the next cultivation at 37°C in atmosphere of 5% CO₂. Results of titration were assessed between the third day and seventh day after infection with appearance of distinct cytopathic changes in monolayer of cells of infected cultures and compared with absence of cytopathic symptoms in non-infected control cultures. The HCV titre calculation followed the Reed and Mench formula⁵.

Results and Discussion

Protection of SW-13 cells from the cytopathogenic effect of HCV.

In control cell cultures infected with HCV the cytopathogenic effect of the virus was observed. Specifically, 25% of SW-13 cells monolayer had been destroyed already three days after the cultures had been infected with 10 TCD₅₀/ml HCV. Previous experiments had shown that peptides 324-337, 333-346 and 343-355 in the concentration range from 0.1 mcg/ml up to 10 mcg/ml do not have cytotoxic effects on SW-13 cells in culture *in vitro*. This allowed the use of this concentration range to investigate the antiviral activity of the peptides.

The addition of peptides 324-337, 333-346 and 343-355 to cell cultures infected with HCV protected the SW-13 cells from the cytopathogenic effect of virus. With all

three peptides, it would be possible to protect the viability of 100% of the SW-13 cells, for 3 days after their infection with 10 TDC₅₀/ml HCV (Table 1). In control cell cultures infected with HCV, by three days they were developing cytopathogenic symptoms which affected 25% of the monolayer. At the same time, in cultures that contained peptides 324-337, 333-346 or 343-355, the cytodestruction symptoms were either less developed or didn't exist at all. The maximum antiviral effect that could keep the cell cultures viable for 3 days after HCV infection was achieved in the presence of 1,0 mcg/ml of peptide 324-337, 0.1 mcg/ml and 10mcg/ml of peptide 333-346 and 10mcg/ml of peptide 343-355 (Table1).

Table 1. Effect of peptides 324-337, 333-346 and 343-355 on the viability of SW-13 cell cultures infected 10 TCD₅₀ HCV (3 days after infection).

	Time of addition of drug	Peptide conc. 0	Peptide conc. 0.1mcg/ml	Peptide conc. 1 mcg/ml	Peptide conc. 10 mcg/ml	Reaferon 5000Units/ml
Peptide 324-337	At time of infection	75	80	100	90	
Peptide 324-337	24 hours after infection	75	75	100	90	
Peptide 333-346	At time of infection	75	100	90	100	
Peptide 333-346	24 hours after infection	75	80	75	75	
Peptide 343-355	At time of infection	75	95	90	100	
Peptide 343-355	24 hours after infection	75	75	75	90	
Reaferon control	At time of infection					100
Reaferon control	24 hours after infection					100

Note: Results are expressed as a percent of viable cells on Day 3 of cell culture after HCV infection

A more visible antiviral effect was observed when the peptides were added to the cell cultures together with the virus than when the peptides were added 24 hours after the cultures were infected with HCV (Table1). When peptides 324-337 (1mcg /ml and 10mcg/ml) and 333-346(10mcg/ml) were added to cells culture 24 hours after infection, the peptides increased the viability of the SW-13 cells infected with HCV to 15-25 % compare to the control infected cell culture (3 days after infection). In the later periods after infection, the cytopathogenic action of HCV was still developing, despite the antiviral effect of peptides. 6 days after infection by HCV, the SW-13 cultures that contained peptides 324-337, 333-346 or 343-355 were dying as much as in control cultures of SW-13. The positive control antiviral drug Reapheron, at the high dose of 5000 units/ml had a more noticeable ability to protect the viability of SW-13 cells. In cultures, that contained Reapheron, there was 100% viability after 3 days and after 6 days of infection by 10 TCD₅₀/ml HCV (Table1).

Reduction of virus titres in cell cultures

The protection of cells from the cytopathogenic effect of HCV is not direct evidence of an antiviral effect of peptides 324-337, 333-346, 343-335. It is known that the absence of a cytopathogenic effect of virus does not mean that active production of virus in cells has stopped. It is typical for HCV that it can develop as a chronic or persistent infection in different types of cells. For a direct assessment of the antiviral effect of peptides 324-337, 333-346 and 343-355, a determination of HCV titre in

SW-13 cell cultures infected with HCV. On Day 3 after the cultures were infected, samples of supernatant were titrated in secondary cultures of SW-13 cells. Table 2 shows the results of titration.

Table 2. Effect of peptides 324-337, 333-346 and 343-355 on the replication of HCV in SW-13 cell cultures infected 10 TCD₅₀ HCV

	Time of addition of drug	Peptide conc. 0	Peptide conc. 0.1mcg/ml	Peptide conc. 1 mcg/ml	Peptide conc. 10 mcg/ml	Reaferon 5000Units/ml
Peptide 324-337	At time of infection	3.2	2.5	2.3	2.0	
Peptide 324-337	24 hours after infection	3.2	3.2	3.3	3.1	
Peptide 333-346	At time of infection	3.2	2.7	2.3	3.0	
Peptide 333-346	24 hours after infection	3.2	3.2	3.6	3.0	
Peptide 343-355	At time of infection	3.2	3.0	2.5	3.0	
Peptide 343-355	24 hours after infection	3.2	2.3	4.0	4.0	
Reaferon control	At time of infection					0
Reaferon control	24 hours after infection					0

Note: Results expressed as the titre of HCV (log TCD₅₀/ml) in supernatants 3 days after infection of the SW-13 cell cultures

In control cultures 3 days after infection, HCV titre reached 3.2 log TCD₅₀. All the peptides under investigation decreased HCV titre in infected cultures. Three days after infection, in the supernatants of cultures that contain peptides 324-337, 333-346 and 343-355 minimum titres of HCV was 2 log, 2.3 log and 2.5 log TCD₅₀/ml (Table 2). Peptide 324-337 suppressed HCV replication at concentrations from 0.1 up to 1mcg/ml and at the same time HCV titre was decreased by 0.7-1.2 log TCD₅₀/ml. Peptides 333-346 and 343-355 had an antiviral effect at concentrations from 0.1 to 1mcg/ml, and decreased HCV titre by 0,5-0,9 log TCD₅₀/ml. The most effective antiviral action of all of the peptides was achieved when they were added into the cell cultures at the moment of infection, not 24 hours after infection.

The result of this research is the evidence that peptides of hinge region of ezrin have an antiviral effect on HCV. The series of peptides mimicking the sequences 324-337 (TEKKRRETVEREKE), 333-346 (EREKEQMMREKEL), and 343-355 (KEELMLRLQDYEE) of the hinge region of ezrin inhibits the replication of HCV and effectively protected human cells *in vitro* from cytopathogenic effect of HCV (table 1 and 2). This data supports the described antiviral effect made by the same peptides in cultures of J-96 and L-41 human cells, infected by encephalomyocarditis virus⁷, and also in the model of lethal mouse infection by Herpes Simplex Virus Type I⁸.

We suggest that the peptides that mimic different parts of hinge region of ezrin, are interacting with the same cell receptor and this may explain why they have similar types of biological activity, and specifically have similar antiviral action *in vitro* and *in vivo*. We have already reported that the peptide 324-337 induces significant changes of mRNA spectrum of interferons and cytokines synthesised by cells J-96¹. It

appears that changing the spectrum of synthesised interferons and cytokines is a real basis for increasing immunity of cells to viral infection. Similar changes are induced by peptides of hinge region of ezrin, in different cell types such as SW-13, J-96 and L-41. These changes become a basis for similar biological effects as inhibition of replication of viruses and protection of cells from the cytopathogenic effects of viruses. As peptides of ezrin can change spectrum of cellular cytokines and interferons, their antiviral action should be effective against different viruses and particularly against viruses responsible for encephalomyocarditis, herpes and hepatitis C.

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