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Original Article

Induction of Antiviral Factors by IFN-α 2a is Time and Dose Dependent

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Abstract

Background and Aims: Interferon alpha is an effective cytokine in viral infections, where it has various roles in immune function. The use of this antiviral agent in the treatment of viral infections and even cancers is common, although, the beneficial effects of this antiviral agent in high doses can be associated with side effects that limit its use. In this project, we tried to investigate the effects of different doses and timing of interferon alpha treatment on the expression of downstream interferon signaling genes and evaluation of the antiviral effects in patients with chronic hepatitis C.

Materials and Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from treatment-naive HCV-infected patients. The cells were treated with different doses of Interferon alpha 2a (IFN-α 2a) and the mRNA expression of target genes (ISG15, MXA, PKR and OAS) at different time points was evaluated by Real Time PCR. The levels of ISG15 and OAS were measured in culture supernatant using ELISA and the level of HCV NS5A in chronic HCV patients was measured by flow cytometry.

Results: Our results showed that IFN-α 2a effect on the expression of antiviral proteins was dependent on dose and time of administrated IFN-α.

Conclusion: This finding indicates that IFN-α should be used at optimal dose to achieve the best efficiency and established balance between antiviral and anti-tumor effects of IFN-α with fewer side effects.

Keywords: Antiviral factors; IFN-α 2a; dose dependent

Introduction

Alpha interferon was discovered several decades ago and is classified as a type I cytokine that includes about 13 sub-groups (1, 2). The main essential function of the interferons is known to be their antiviral activity (3).

Other functions, such as anti-tumor activity, increase of MHCI antigen expression, enhancement of NK-cell activity and CD8 + cytotoxicity have also been observed, as well as immunosuppressive activities, such as immunomodulatory effects in various cells of the body and cell cultures (4-6). The use of this cytokine in the treatment of several blood malignancies such as hairy cell leukemia and chronic myeloid leukemia also has shown therapeutic benefits. It is also common in the treatment of metastatic melanoma and viral diseases, such as chronic hepatitis C (7-9).

Chronic hepatitis C is a health problem that

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affects around 71 million people in the world (10, 11). The prevalence of this disease is 1.6% and the annual mortality rate is 400,000 people from cirrhosis and hepatocellular carcinoma (12).

The traditional treatment of chronic hepatitis C is the use of interferon alpha and ribavirin, a new generation of medicines has been used as the direct acting antiviral (DAA) with better efficiency than interferon/ribavirin, but these drugs are less available and expensive in some endemic regions (13) .

Also, many works have been done in recent years in animal tumor models and have achieved acceptable results in the clinical use of alpha interferon. The importance of different mechanisms in response to interferon alpha therapy is still a debatable issue (14,15).

Interferon alpha signals is via the JAK-STAT pathway by binding of interferon to its receptor and phosphorylation and activation of JAK1 and TYK2, which starts the signaling pathway and the formation of the ISGF3 complex (16). Transmission of the complex to the nucleus and binding to the ISRE (Interferon Stimulated Response Element) induces the transcription of the ISGs, MXa, PKR, and OAS genes (17, 18). In this study, the expression level of ISG15, MXa, OAS and PKR and antiviral effect at different doses and time intervals of interferon alpha treatment was measured in PBMCs of patients with chronic HCV genotype 1a.

Methods

PBMC culture. Serum samples were collected from 10 infected patients with chronic HCV genotype 1a before treatment (Razi Laboratory in Qom, IRAN). PBMCs were isolated and cultured in 96-well plates according to the standard protocol in RPMI 1640, 5% CO_2 and 37 ° C. The cells (10^5) were then treated with the recombinant IFN-Alpha 2a (R & D SYSTEMS) protein for different times with 24 hrs intervals according to the values of Table 1. The level of toxicity of IFN-α 2a protein and cell viability were tested and verified by MTT assay (Cells isolated from 10 patients were divided into different groups and each group consisted of 10 samples).

Table 1. Dose of interferon alpha treatment in different experimental groups of cultured PBMCs

Group	IFN-α 2a
Group 1	**1ng/ml**
Group 2	**5ng/ml**
Group 3	**10ng/ml**
Group 4	**50ng/ml**
Negative Control Group	**-**

RNA extraction and reverse transcription. At different time points after IFN-α 2a treatment, RNA was extracted using the commercial kit (TOTAL RNA Prep kit BIOFACT, Korea) according to the manufacturer's protocol.

The extracted RNA was measured and immediately reverse transcribed using RT PCR kit according to the manufacturer's instructions (BioFact, Korea) and cDNA was kept at -20°C.

Table 2. Primers of IFN-α downstream genes designed for real time PCR

Primer	Forward (5'-3')	Reverse (5'-3')
PKR	CTCCGCACCGCTCTTCCTC	AATCGGTGTTTTCCCTGGCTTAG
ISG15	GCTCCATGTCGGTGTCAGAG	AGGTTCGTCGCATTTGTCCA
MX1	AGGAGATCGGTTCTGGGTC	TGCAAAGTAAGCTTGGAATGG
OAS1	TTCCGCATGCAAATCAACCA	GGAGCCACCCTTTACCACCTT
HPRT1 (Internal control)	**CCCTGGCGTCGTGATTAGTG**	**ACCCTTTCCAAATCCTCAGCATA**

Real time PCR. The relative expression level of the IFN-α downstream genes were evaluated compared to untreated control group (Livak method (19) using real time PCR with the designed primers for target genes (Table 2).

Real time PCR was performed using RealQ PLuS Master Mix Green mixer (Amplicon, Denmark) according to the proposed standard

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manufacturer's time schedule and annealing temperature of 60°C.

Measurement of OAS and ISG15 using ELISA. The OAS and ISG15 levels were measured in the supernatants of cultured PBMCs, isolated from infected patients with chronic HCV, using the ELISA kit (Fine Biotech Company) according to the instructions of the manufacturer. Briefly, the samples and standards were added in the duplicates and after 90 min incubation (37°C) were washed. Then the biotin-detection antibody was used, which was followed by streptavidin-HRP.

Finally, the substrate 3, 3′,5, 5′-tetramethyl benzidine (TMB) was added and sulfuric acid was used to stop the reaction. The absorbance was read at 450 nm using ELISA reader (Stat Fax 2100).

Detection of HCV NS5A antibody by Flowcytometry. In a 96-well plate, PBMCs from HCV-infected patients were cultured and incubated with different concentrations of IFN-α 2a. After 96 h, cells were collected and washed 3 times with FACS buffer (PBS containing 2% FBS). Primary anti-HCV NS5A antibody (Abcam) was used at a 1:50 dilution and incubated with cells (30 min). Secondary antibody)Goat Anti-Mouse IgG1 heavy chain: FITC, Abcam) was used at a 1:100 dilution and incubated for 30 min, then the cells were washed 3 times with FACS buffer and analyzed by BD FACS CANTO (10^4 events per sample). The test was repeated twice and Data was analyzed by FlowJo software.

Results

Expression pattern of target genes. The results showed that the mRNA expression level of the measured antiviral genes was increased depending on the dose of IFN-α2a. The mRNA expression was the highest at 72 h after treatment with all tested doses in accordance with Table 1 (Fig. 1).

At two initial doses, the expression level of antiviral factors was increased, and in two higher doses, reduction of expression was observed compared to the first two groups due to saturation of interferon receptors in accordance with Table 1 (Fig. 2).

IFN-α 2a induced expression of ISG15 and OAS. Our results demonstrated higher levels of protein expression ISG15 and OAS by PBMCs after the treatment with different doses of IFN-α 2a.

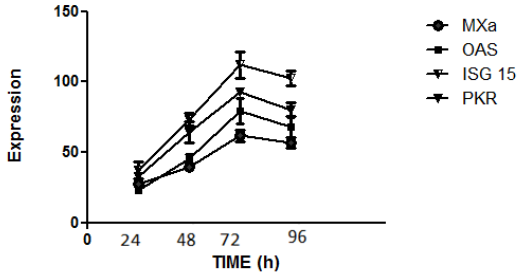


Fig. 1. Expression pattern of OAS, MXA, PKR and ISG15 mRNAs at 24, 48, 72 and 96 hours of IFN alpha treatment. The expression level of mRNA showed that the highest mRNA expression of antiviral protein was observed at 72 hours after treatment with IFN-α2a protein in all the tested doses.

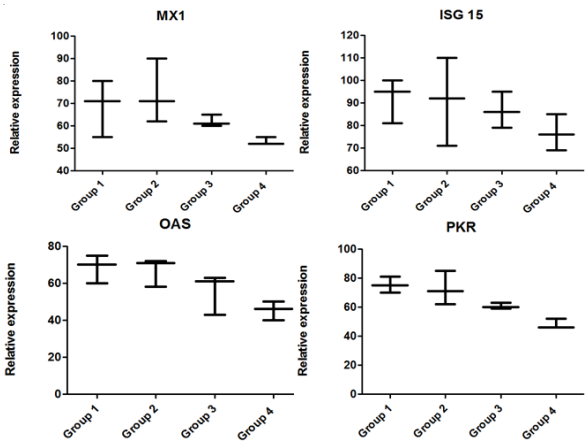


Fig. 2. The level of expression of PKR, MX1, OAS, and ISG15 genes was measured in groups treated with different doses of IFN. It was shown that 1 and 5 ng concentrations increased the antiviral proteins in comparison with other groups, and in higher concentrations (group 3 and 4) due to its toxicity, a reduction was observed in the expression of antiviral proteins (Livak method, Real Time PCR).

We also observed that these changes were related to the dose of IFN-α 2a (Fig. 3). The results demonstrated a significant up-regulation of the OAS and ISG15 levels in studied groups compared to control group.

HCV–NS5A antibody level in different test groups. Cells cultured with different concentrations of IFN-α 2a were collected after 96 h of treatment and the HCV NS5a antibody level was determined by Flowcytometry (Fig. 4). The results showed that there is a significant

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difference between four treatment groups and at lower doses, the antiviral activity of interferon is higher in these samples and vice versa.

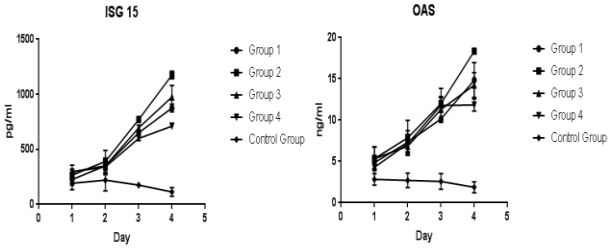


Fig. 3. The levels of OAS and ISG15 proteins in PBMCs after treatment with IFN-α 2a. The PBMC were isolated from treatment-naive HCV-infected patients and cultured in 96-wells plate in the presence of different concentrations of IFN-α 2a for 96 h. The levels of OAS and ISG15 were measured in the culture supernatants by ELISA.

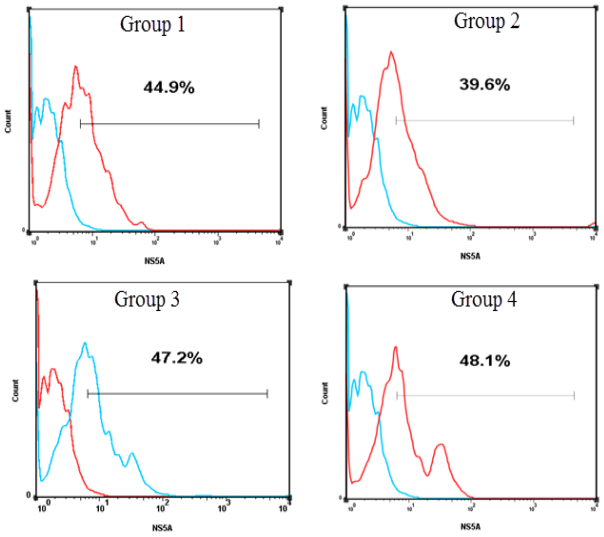


Fig. 4. Flow cytometery detection of NS5A expression level in HCV-infected cells after treatment with IFN-α 2a. Cells from different groups were treated with IFN for 96 h (5ng/ml) and the cells were collected and analyzed after interaction with specific antibody. The histogram plots show the percent of HCV-NS5a expression level in different groups compared with negative control.

Group 2 had the greatest reduction in antibody level among all groups as determined by Flow cytometry (Fig. 5).

Discussion

The present study investigated the effect of IFN-α 2a on the expression pattern of ISG15, OAS, MX1 and PKR in PBMCs which isolated from HCV-infected patient volunteers.

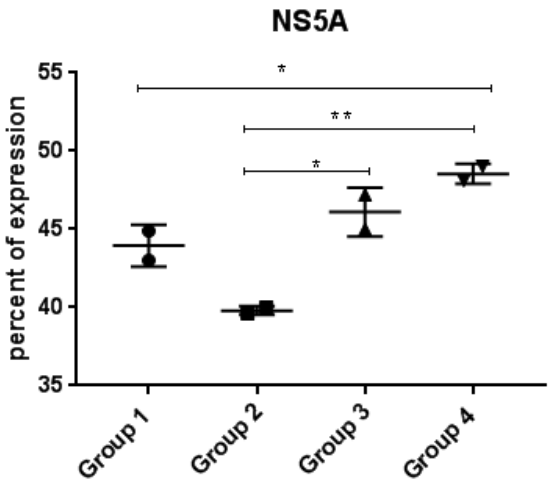


Fig. 5. Comparison of the NS5A expression level in different treatment groups compared with negative control. The percentages of NS5A expression level obtained by flow cytometry were compared by GraphPad software using One-way ANOVA (post hoc with Duncan's correlation) method. Results with P values of <0.05* and <0.01** were considered statistically significant.

Our results demonstrated that the expression patterns of ISG15, OAS, MX1 and PKR were approximately similar in all experimental groups, however, their levels were different. The mRNA levels of mentioned gene were increased after 72 h following administration of IFN-α 2a. Our data also demonstrated that protein levels of ISG15 and OAS were increased after 96 h and the antiviral effects of the genes and proteins stimulated indicate differences in these effects in different groups by flow cytometry.

Studies in recent years indicated that ISG15, OAS, MXA and PKR not only have an antiviral effect, but also demonstrated their various functions (20). ISGylation has been reported to interfere with lung cancer cells and as a result regulates the reproduction of ISG (21). Polymorphisms of OAS1 have been reported in type I diabetes, which is associated with viral factors (22). PKR is a regulator of translation and also involves in various signaling pathways, including TLR4-associated apoptosis, NF-Kβ transcription factor activation, induction of inflammatory signaling, CD8+ functional regulation and IgE class switching (23-25). Due to the insufficient knowledge about the obvious function of this

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signaling complex, more investigations are needed to evaluate the effects and function of kinases, STATs and other puzzles of these pathways in different subtypes of IFN-α and their precise clinical applications (26).

Previous studies have shown that gene expression induced by IFN is mainly dependent on dose and time, and the expression level of these proteins can be changed based on capacity of the cell and the initial dose of IFN-α 2a (26-28). IFN-α has the capacity to induce many antiviral proteins in infected cells, such as threonine kinase, activated dsRNA, 2.5 OAS, and viral immunity mediators of MXA and ISG15.

IFN-α induces signaling and coherent activity that increases the expression of the MHC-1 antigen and induces antiviral activity (29). Interferon type 1 also has immunomodulatory function such as induces NK cell and CD8 + cytotoxic activity, Th1 responses, positive regulation of MHC1 expression on the surface of the cancer cells to increase antigen presentations and apoptotic cell mediation (30, 31). In the present study, we observed that the treatment with IFN-α at different concentrations could have different effects, and the further increase in the dose of interferon does not lead to an increase in the expression of antiviral proteins. Also, the protein levels of antiviral proteins were different at various time points of 24, 48, 72, and 96 h and mainly dependent on the dose of IFN-α 2a. We observed the highest mRNA levels at 72 hours after the addition of IFN-α 2a protein.

The increasing expression of antiviral factors (or their genes) indicates a progressive increase in antiviral proteins, which reached its peak at 72 h, and subsequently a decrease in expression levels at 96 h. Also, the highest levels of anti-viral proteins ISG15 and OAS were seen at 96 h and antiviral effects of IFN-α 2a on HCV were also measured at 96h by flowcytometry too (it is not clear what do you mean by this sentence). In the results, Group 2 has the highest expression level of the mRNA and protein targets, with the highest reduction of NS5a antibody expression, which is due to more antiviral effects than other groups.

Various rates of expression of antiviral proteins in previous studies have been also reported, depending on the cell type, number of cells and doses used in different hours. For example, the level of expression of antiviral proteins in Huh-7 cells treated with IFN-I (10 ng / ml) reached its peak after 48 h (32). It has also been shown that using a low level of IFN-α for several days can show the full function of antiviral effects *(this sentence is difficult to understand, please try to re-write)* (32, 33). In fact, the biological effects of IFN-α, not only depended on the dose, but also depended on the IFN-α receptor expression on cells (16, 32, 34).

It has been shown that IFN-α subtypes have different antiviral effects in viral infections, as well as their side effects and patient tolerance in clinical studies. Even the type of antiviral proteins, which are induced following viral infection by each of the antiviral subtypes (IFN- α) is different, for example, IFN-α5 induces high expression of OAS1, while IFN-α2 and IFN-α5 subtypes induced the expression of ISG15 (27, 35). This observation is consistent with our study, here we showed that expression of ISG15 among the other antiviral proteins, which were measured in this study, was higher than other anti-viral proteins following treatment with IFN-α 2a that indicates this subtype mainly exert its effect through induction of ISG15.

Conclusion

According to these results, the use of IFN-α as an antiviral and anti-tumor agent probably depends on the type of target cells, as well as the dose and time of administration.

So, to improve efficacy of IFN-α 2a and its use in clinical application, we should obtain an optimal dose and time of administration to avoid side effect and achieve the best result.

Acknowledgment

Not applicable

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Conflict of interest

The authors declare that they have no conflict of interest.

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