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Original Article

The Effect of SB431542 TGF-β Receptor Inhibitor, on HCV Replication in PBMCs of Patients with Chronic Hepatitis

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Abstract

Background and Aims: TGF-β is an effective cytokine in the viral replication cycle, which is also highly relevant to the pathogenesis of some viral infections. TGF-β induction by viral proteins is one of the ways to escape the virus from the immune system by inhibiting interferon signaling and other immune system factors. In recent years, the role of TGF-β and its inhibitory signaling has been confirmed in clinical trials. In this study, using SB431542, a TGF-β type I activin receptor like kinase inhibitor, the effect of TGF-β reduction on IFN expression and antiviral activity in PBMCs of patients with chronic hepatitis C was investigated.

Materials and Methods: PBMCs from 10 patients with chronic hepatitis and 5 healthy individuals were isolated with Ficoll solution and cultured in the presence of different concentrations of sb431542. RNA was extracted at different time points of culture, with Qiazol lysis reagent and cDNA was synthesized with commercial kits. The relative expression levels of TGF-β, IFN-α and HCV Core mRNA were determined using with REAL TIME PCR. The expression level of TGF-β protein was also measured in supernatant of cultured cells by ELISA method.

Results: The inhibitory effect of different concentrations of SB431542 at various time point showed that it peaks 48h after treatment and the maximum increase of IFN-α expression and significant antiviral effects reached 72 h after treatment.

Conclusions: The effects of TGF-β on IFNs may be correlated with inverse ratio depending on the level and duration of expression, which indicates the regulatory role of these proteins in the immune system against the viruses. TGF inhibitory drugs can augment immune system against the viruses.

Keywords: TGF-β, IFN-α, SB431542.

Introduction

HCV is a single-stranded RNA virus with positive polarity which has a genome length of 9.6KB encoding a polyprotein(1). This polyprotein is processed by viral and cellular proteases, and produces 11 proteins with different functions on cellular pathways (2). HCV is a major contributor to the development of chronic liver disease, with around 71 million people diagnosed with chronic hepatitis C that are vulnerable to advanced liver disease (3, 4). HCV replicates mainly in hepatocytes but other cells like PBMCs supports virus replication at low level (5, 6). Like many other pathogens, immune system affects the virus replication and pathogenesis (7, 8). TGF-β is a cytokine with various functions that have different effects on many cellular pathways signaling and can be referred to as immune regulator (9, 10).

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One of the important roles of this cytokine is its effect on the replication of viruses (11, 12). For example, TGF-β increases the proliferation of HCV, upon its induction by Core and NS5 proteins (13, 14). On the other hand, TGF-β inhibits IFN-α and IFN-λ signaling and induces expression of FOXO3a and SOCs, which provide conditions for virus replication (15, 16).

SB431542 is an inhibitory molecule for the TGF-β signaling pathway, which, by effecting on TGF-β kinase receptors, inhibits the TGF-β induced transcription (17, 18). The clinical function of SB431542 has been demonstrated by blocking tumor cell invasion, angiogenesis and metastasis as a complementary therapeutic agent in human cancers (19, 20). In this study, the effect of SB431542 on the level of HCV replication in PBMC cells of patients with chronic hepatitis C and its inhibitory role on the replication of the virus were investigated.

Methods

PBMCs Culture. PBMCs were isolated from the peripheral blood sample of 10 untreated patients with chronic hepatitis C, genotype 1a, by the Ficoll solution (GE healthcare life sciences). Five samples of healthy individuals were also used as control (which had not been reported any infectious disease during the last 3 months). Cells were cultured using a standard protocol in the RPMI 1640 media (105), 5% CO_2, at 37 °C.

The percentage of cell viability was measured by MTT test for different doses of SB431542 (Sigma-Aldrich) from 0.5 to 10 μM. Then cells cultured in a 96 wells plate were treated with SB431542 at concentrations of 0.5, 1.5, 10 μM along with untreated cells as control.

Real Time PCR. At 24, 48, 72 and 96h after treatment, extraction of RNA was performed using QIAzole solution according to the standard protocol. After evaluating the concentration and quality of RNA, cDNA synthesis was performed using the Biofact kit according to the manufacturer's protocol. Real Time PCR method was done with designed primers (Table 1). Real time PCR was per-formed using with 5X HOT FIREPOL EVA GREEN QPCR MIX PLUS with ROX (Solis BioDyne, Estonia) in STEP ONEPLUS (ABI) device. The reaction was performed for 40 cycles of 95 °C 15s, 60 °C 20s, and 72 °C 20s. The results were calculated using Livak method by considering HPRT as internal control.

ELISA for TGF-β. Supernatant of HCV infected cells cultured for 72h at the presence of SB431542 were collected and applied for the measurement of TGF-β expression. TGF-β expression was measured using a human TGF-β ELISA kit (eBioscience) and according to the manufacturer's protocol. The protein concentration was quantitated by ELISA plate reader using standard curve.

Statistical Analysis. The results were analyzed using one way ANOVA and T tests method with Graph pad v7.3 software.

Results

Cell viability. Percentage of PBMCs viability under the treatment with SB431542 was monitored in an MTT test at concentrations of 0.5, 1, 5, and 10 μM. The results obtained at 24, 48, 72 and 96 hr after treatment indicated that the viability remained above 75% till 96 hours after treatment at the treated concentrations, which was taken as appropriate time for subsequent experiments (fig 1).

mRNA real time expression for TGF-β1 and IFN-α. The level of mRNA expression of TGF-β and IFN-α in PBMCs after treatment with different concentrations of SB431542 was analyzed in comparison with an untreated control group (Fig 2, 3). Then, HCV-core

Table 1. Primers designed in this project for Real time PCR.

Primer	Forward	Reverse
HCV-core	5′-GCACGAATCCTAAACCTCAAAG	5′-GCGCGGCAACAAGTAAAC
TGF-β	5′-GCAACAATTCCTGGCGATACC	5′-AGTGAACCCGTTGATGTCC
IFN-α	5′-CTCAAGCCATCTGTGTCCTCC	5′-CTACCACCCCCACCTCCTGT
HPRT (Internal Control)	5′-CCCTGGCGTCGTGATTAGTG	ACCCTTTCCAAATCCTCAGCATA′-5

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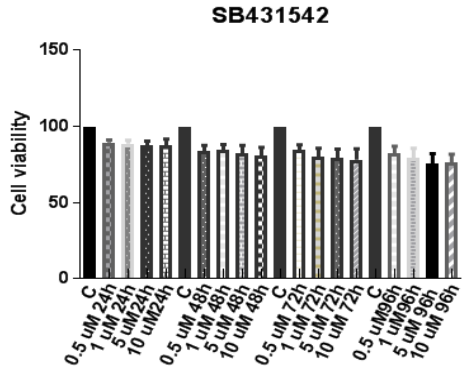

Fig. 1. The results of the MTT test at different concentrations and time points of cells treated with sb431542.

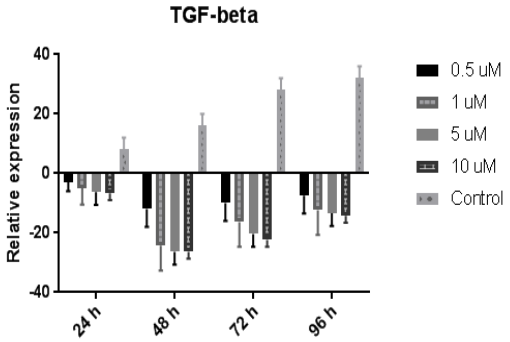

Fig. 2. Relative expression of TGF- β mRNA in treated and untreated groups. There was a significant difference in the expression of the treated groups at different concentrations and hours compared to the control group (P <0.001 ***). Among the groups, the concentration of 0.5 μM was significantly different from the concentration of 1 μM (P <0.05 *) and concentration of 5 and 10 μM (P <0.01 **) in 48h after treatment.

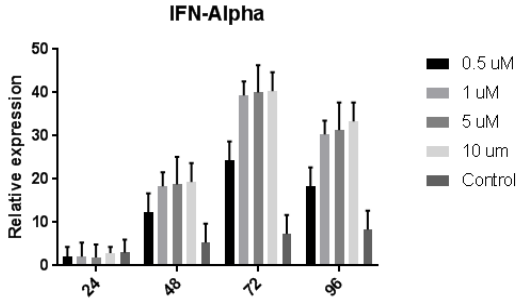

Fig. 3. The expression pattern of IFN-α in the studied groups. No significant difference was observed between the treated groups and the control group at 24 h. At 48 h in groups 1, 5 and 10 μM, there was a significant difference with control group (P <0.01 **). At 72 and 96 h, there was a significant difference between treated and untreated groups (P <0.001 ***). There was a significant difference between the concentrations of 0.5 μM and other treated groups in 72 and 96h (P <0.01 **).

relative expression comparing to untreated PBMCs was calculated to evaluate the anti-viral effects of SB431542 in treated groups as compared to control group (Fig 4).

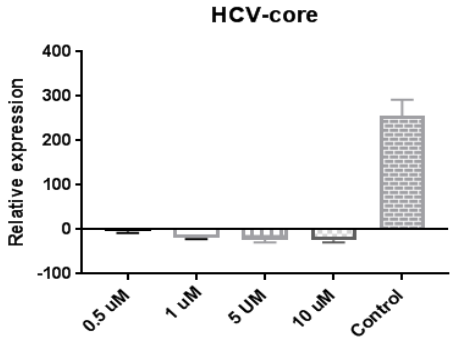

Fig. 4. Expression level of HCV-Core in the treated groups compared to control group. The level of expression of the gene was measured at 72h after treatment; the expression level of HCV-Core in the treated groups was significantly different from the control group (P <0.001 ***).

TGF-β protein expression. Measurement of TGF-β protein expression was assessed to evaluate the SB431542 function in TGF-β expression. Based on the real time results, the cells treated with 1 μM of SB431542 for 72 h as the best group for TGF reduction was chosen for ELISA evaluation. The expression level of TGF-β compared to the control group was statistically significant, which indicated that TGF-β expression was inhibited in treated groups at 72h after usage (fig 5).

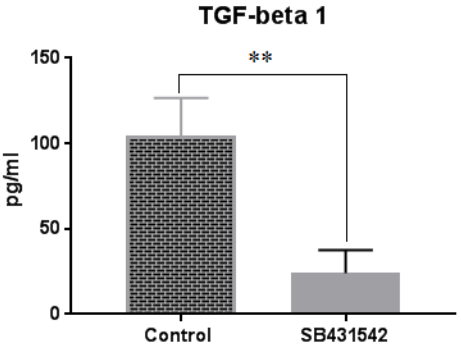

Fig. 5. Measure of expression TGF-β in target groups. The level of expression of TGF-β in the untreated control group was statistically significant compared to the treated group with SB431542 (P <0.01**).

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Discussion

The role of TGF-β as an important cytokine in the mechanism of pathogenesis of some diseases has been proven (21). Inhibition of TGF-β has been shown to be an effective strategy for complementary treatment of cancer (22). SB431542 is one of the TGF-β inhibitors, which has been classified as small molecular inhibitors that prevents TGF-β induced transcription, apoptosis and growth suppression through inhibition of TGF-beta receptor kinase (19).

Increasing the expression of TGF-β in viral infections has been clearly seen, indicating the importance of this cytokine in the life cycle of the some viruses (15, 23). Xia et al showed that TGF-β induced by the RSV, RV, and IAV viruses is reduced by inhibitors such as SB431542 (24). Thornburg et al showed that the use of TGF-β inhibitors such as SB431542 can reduce the proliferation and pathogenesis of Ebola virus in human hepatocytes ,which these effects observed in controlling the level of phosphorylation of PI3K, AKT and PKC by TGF-β(25). Li and colleagues have shown that TGF-β, by increasing cellular adhesion molecules in influenza-infected cells, enhances the host's sensitivity to bacterial coinfection, which indicates the role of TGF-β in the pathogenesis of this virus and the use of inhibitors of this cytokine in order to take advantage of their clinical benefits showed promising results (26).

In this study, the effect of different concentrations of SB431542 on inhibiting TGF-β signaling was investigated and the PBMCs of patients with chronic hepatitis C infection were used as a source of virus replication and increased TGF-β expression level.

It was observed that at 24 hours after treatment with 0.5, 1, 5 and 10 μM concentrations of SB431542, the inhibitory effects began and peaked at 48 h. At 72 and 96 hours after treatment, the inhibitory level was reduced. In this experiment, due to the effects of TGF-β on IFNs signaling and inhibition of IFN expression, IFN-α expression levels were measured after treatment with SB431542. The level of expression of IFN-α in the presence of TGF-β inhibitor in treated groups did not have a significant difference at 24 h compared with control group while, 48h after treatment, the expression level of IFN was observed to increase and peak at 72h and then declined at 96 h. In other word, the SB431542 inhibitory effect on TGF-β appeared to peak at 48 hours after treatment, and subsequently, an increase in the expression level of IFN occurred at 72h.

Antiviral effects in infected cells at 72 hours after treatment with SB431542 were evaluated due to the peak of IFN-α expression in this time. Reduction in the expression level of HCV-Core in treated groups was -3.8 to -24.36 times more than the control group, there was a significant difference between the control group and treated group, but there was no significant difference between treated groups. NS5a protein expression level was also analyzed by flow cytometric method which was statistically significant compared to the control group (its data was not reported).

Among the measured concentrations, the concentration of 0.5 μM had lower potency in alternation of the tested parameters and no significant difference was observed between the other concentrations. According to the results of the MTT test and Real Time PCR, We selected 1μM of SB431542 to the measurement of TGF-β by ELISA and observe that its expression reduced significantly compared to control group.

The effects of SB431542 on the PBMC of patients with chronic hepatitis C in this study revealed a reverse relationship between TGF-β and IFN-α in expression at different hours after treatment, which shows the effects of these two proteins on signaling of each other.This effects that like a double-edged sword can regulate the balance of virus and immune system, might be controlled by TGF-β inhibitors in clinical studies and therapies.

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