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CLINICAL STUDY

FOXP3, RORγt and IL-10 cytokine profile in chronic heart failure

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ABSTRACT
BACKGROUND: Distinct subsets of T cells play crucial regulatory roles in inflammatory processes of chronic heart failure (CHF). Retinoic acid receptor-related orphan receptor-γt (Ror-γt) and Forkhead box P3 (Foxp3) have been defined as the "master regulators" of Th17 cells and Treg cells, respectively. At the same time, anti-inflammatory cytokines such as IL-10 may neutralize inflammation in CHF. The current study was designed to compare FOXP3, RORγt and IL-10 protein expression in the blood and IL-10 in supernatant PBMCs in CHF patients versus normal subjects.
PATIENTS AND METHODS: Our study population consisted of 42 patients with CHF in four different function classes and 42 healthy subjects who served as controls. RNA extraction and cDNA synthesis was performed and mRNA expression for genes FOXP3, RORγt, IL-10 was determined by RT-PCR. The amount of IL-10 protein in supernatant of PBMCs was measured by ELISA technique.
RESULTS: There was no significant difference in FOXP3, RORγt, IL-10 protein expression and supernatant PBMCs IL-10 in CHF patients as compared to control. The level of Foxp3 was significantly lower in CHF patients with ischemic vs non-ischemic cause (p = 0.04).
DISCUSSION: Although inflammation plays a central role in the pathophysiology of CHF, the roles of FOXp3, RORγt, and IL-10 remain to be determined *(Tab. 3, Ref. 33)*. Text in PDF *www.elis.sk.*
KEY WORDS: CHF, inflammation, FOXP3, RORγt, IL-10.

Introduction

Heart failure (HF) is one of the most common cardiovascular diseases, which represents a major public health burden (1, 2). Congestive heart failure (CHF), as a multifaceted clinical syndrome, is no longer merely a pump failure of the heart but a multisystem disease which affects humoral, neuroendocrine, renal and musculoskeletal systems in addition to the cardiovascular system (3). Therefore, the pathophysiology of CHF is exceedingly complex and multidimensional. Over the decades, it has been shown that many features of CHF can be explained by the known biological effects of inflammatory mediators. As follows, an increased level of circulating pro- and anti-inflammatory cytokines has been expressed in its pathogenesis (4). In addition to cytokine's effects on myocardial remodeling, cytokines have been shown to affect both the myocyte contractility and the extracellular matrix and impact myocardial function (5). By representing the significance of inflammation markers in heart failure by previous studies, a novel immunological-inflammatory model is required to explain the development and progression of heart failure (6). Various inflammatory cytokines produce a myriad of effects and cause imbalance in cardiac homeostasis (4). T helper (Th) cells, also known as CD4 T cells, play an important role in orchestrating adaptive immune responses to different antigens (7).

FOXP3+CD4+CD25+ Regulatory T (Treg) cells and IL-17 producing helper T cells (Th17) are critical subsets of Th cells which play indispensable roles in immune homeostasis (8). Naive Th cells stimulated with antigens (Ags) upon activation of T cell receptor (TCR) and TGFb cytokine-mediated signaling, upregulate expression of the transcription factor (Forkhead box P3) FoxP3 and the phenotypic cell surface marker CD25 and therefore develop into distinct Tregs (9). Treg cells exhibit effective suppressive function against pathogenic self-reactive T cells and maintaining immune tolerance (10). In contrast, naive T cells which

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Tab. 1. Demographic data of CHF patients and control group.

	CHF patients (n=42)	Control (n=42)
Age (years)	55±14.1	51.5±12.02
Gender (m/f)	23/22	28/14
IHF/NIHF*	29/13	---------

*IHF=Ischemic Heart Failure. NIHF=Non-ischemic heart failure

stimulated with Ags upon activation of TCR and both TGFb and IL-6 cytokines-mediated signaling, upregulate the transcription factor retinoic acid receptor-related orphan receptor-γt (ROR γt) and consequently develop into Th17 cells (11).

In general, cytokines are interwoven and regulated through a feedback mechanism, which proposes the role of anti-inflammatory cytokines in CHF (12, 13).

Interleukin 10 (IL-10), an important negative regulator of pro-inflammatory cytokines, has potent suppressor properties in macrophages, T-cells and B cells (14). IL-10 has been shown to inherit an important role in the cytokine network and act as a downregulator of cell-mediated immune reactions in CHF patients (15, 16).

In the present study, we sought to evaluate the expression of genes including FOXP3, RORγt, IL-10 and measurement of IL-10 in peripheral blood mononuclear cell (PBMC) culture in CHF patients compared with healthy control subjects.

Materials and methods

Study population

Our study population consisted of 42 patients with CHF and 42 healthy subjects (individuals without coronary artery disease or heart failure) who served as controls. CHF patients were classified as ischemic heart failure (IHF) and non-ischemic heart failure (NIHF) based on their history of myocardial infarction or significant coronary artery stenosis. The characteristics of the study subjects are summarized in Table 1. Patients in the CHF group were enrolled during hospital admission for decompensated heart failure. All subjects underwent a physical examination and answered a standardized questionnaire to assess their medical history, current illnesses, and any medication they were taking. The diagnosis of CHF was based on clinical history, physical examination, electrocardiography, echocardiography and chest X-ray.

All patients were stable during the last month of the study without any change in medication. Exclusion criteria included all conditions that might influence the immune system, such as a recent or current infection, autoimmune disorder, allergic disease, malignant disease, advanced liver disease, renal failure, malnutrition and steroid therapy (within 3 months). Informed consent was obtained from each patient. CHF patients were classified into 4 groups according to New York Heart Association Class I to IV (17). The study was approved by the Ethical Committee of Tehran University Heart Center Hospital.

Hematologic analysis

Blood samples were obtained from all the subjects in recumbent position with a 21-gauge needle for clean venipuncture of an antecubital vein. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient for analysis of real time-polymerase chain reaction (RT-PCR), and cell culture was obtained after centrifugation and stored at - 80°C. Foxp3, RORγt, and IL-10 expressions were measured by RT-PCR. Total RNA was extracted with phenyl chloroformate extraction (cinaclone) according to the manufacturer's instructions. The cDNA was synthesized using random hexamer primers and RNase H-reverse transcriptase (Cinaclone). TaqMan primers and probes for human RORγt, Foxp3 and IL-10 were purchased from ABI Company; all reactions were performed using the Prism 23. For each sample, the mRNA expression level was normalized to the level of actin housekeeping genes.

For cell culture, freshly isolated PBMCs were stimulated with PHA (15 μg/ml) at the density of 2 * 106/ml in complete RPMI1640 with 10 % heat-inactivated fetal calf serum (Gibco BRL, USA) for 48 hours. Then the supernatant was collected and stored at –80 °C for the detection of IL-10 by ELISA. In each group, some samples have been excluded due to technical errors.

Statistical analysis

The data were analyzed by non-parametric methods to avoid assumptions about the distribution of the measured variables. For comparisons of groups, independent T test and ANOVA were used. All values are reported as mean ± SD. Analyses were performed using the SPSS 19 statistical software package program and p values of 0.05 or less were considered significant.

Results

Foxp3 expression in CHF patients

The mean level of Foxp3 expression were lower in the CHF patients (1.78 ± 2.05) than in the control group (1.67 ± 2.28), however the difference was not significant ($p > 0.5$) (Tab. 2).

The mean level of Foxp3 in CHF patients with ischemic etiology was 1.20 ± 1.32; whereas the mean level of Foxp3 in CHF patients with non- ischemic etiology was 3.15 ± 2.79; the difference, however, is not significant ($p > 0.5$) (Tab. 3).

The mean level of Foxp3 in CHF patients with function classes I, II, III and IV were 1.81 ± 1.64, 1.25 ± 1.28, 1.61 ± 2.18, and 2.31 ± 2.57, respectively. However, the difference between groups was not significant ($p > 0.05$) (Tab. 3).

RORγt expression in CHF patients

The mean level of RORγt expression was 1.61 ± 2.13 in CHF patients and 1.62 ± 1.98 in the control group. The difference be-

Tab. 2. Comparison of Foxp3, RORγt, IL-10 and Il protein in PBMC in CHF patients and normal subjects.

	CHF patients	Control	p
Fox p3 level (mean±SD)	1.78±2.05 (n=37)	1.67±2.28 (n=27)	0.84
RORγt Level (mean±SD)	1.61±2.13 (n=28)	1.62±1.98 (n=29)	0.9
IL-10 Level (mean±SD)	0.82±1.2 (n=26)	1.05±1.93 (n=30)	0.61
IL-10 Pr* in PBMC (mean±SD)	399.64±256.97 (n=34)	319.17±199.46 (n=34)	0.15

*Pr = protein

Tab. 3. Comparison of Foxp3, ROR, IL-10 and IL-10 Pr* in PBMC in CHF ischemic/non-ischemic patients and different function classes.

	IHF	NIHF	Pvlaue	Function Class				p
				I	II	III	IV	
Fox p3 level (mean±SD)	1.20±1.32 (n=26)	3.15±2.79 (n=11)	0.04	1.81±1.64 (n=4)	1.25±1.28 (n=10)	1.61±2.18 (n=10)	2.31±2.57 (n=13)	0.6
RORγt Level (mean±SD)	1.16±1.66 (n=21)	2.96±2.89 (n=7)	0.16	1.75±1.71 (n=5)	1.63±1.97 (n=7)	0.95±2.14 (n=7)	2.04±2.62 (n=9)	0.8
IL-10 Level (mean±SD)	0.69±0.72 (n=19)	1.38±2.02 (n=7)	0.41	1.82±3.01 (n=3)	0.53±0.91 (n=4)	0.66±0.77 (n=9)	0.79±0.9 (n=10)	0.51
IL10 Pr* in PBMC (mean±SD)	419.43±247.71 (n=25)	344.68±289.29 (n=9)	0.46	292.9±193.4 (n=6)	613.5±166.1 (n=7)	399.4±283 (n=11)	314.2±250.2 (n=10)	0.06

*Pr = protein

tween two groups was not significant ($p > 0.5$) (Tab. 2). The mean level of RORγt in CHF patients with ischemic etiology was 1.16 ± 1.66 whereas mean level of RORγt in CHF patients with non-ischemic etiology was 2.96±2.89; however, the difference is not significant ($p > 0.5$) (Tab. 3).

The mean level of RORγt in CHF patients with function classes I, II, III and IV were 1.75 ± 1.71, 1.63 ± 1.97, 0.95 ± 2.14, and 2.04 ± 2.62, respectively. However, the difference between groups was not significant ($p > 0.05$) (Tab. 3).

IL-10 gene expression in CHF patients

The mean level of IL-10 expression was lower in CHF patients (0.82 ± 1.2) than in the control group (1.05 ± 1.93), however, the difference was not significant ($p > 0.5$) (Tab. 2). The mean level of IL-10 in CHF patients with ischemic etiology was 0.69 ± 0.72, whereas the mean level of IL-10 in CHF patients with non- ischemic etiology was 1.38 ± .02; the difference, however, is not significant ($p > 0.05$) (Tab. 3).

The mean level of IL-10 in CHF patients with function classes I, II, III and IV were 1.82 ± 3.01, 0.53 ± 0.91, 0.66 ± 0.77, and 0.79 ± 0.9, respectively. However, the difference between groups was not significant ($p > 0.05$) (Tab. 3).

IL-10 protein in PBMCs of CHF patients

We compared the levels of IL-10 in the supernatant of PHA-stimulated PBMCs between two groups of CHF patients and normal subjects. The mean level of IL-10 protein in the culture supernatants of PHA-stimulated PBMCs was 399.64 ± 256.97 in CHF patients and 319.17 ± 199.46 in the control group. However, the difference between two groups was not significant ($p > 0.5$) (Tab. 2).

The mean level of IL-10 protein in PBMC of CHF patients with ischemic etiology was 419.4 ± 247.7, whereas that of IL-10 protein in PBMC of CHF patients with non-ischemic etiology was 344.7 ± 289.3. The difference between two groups was not significant ($p > 0.5$) (Tab. 3).

The mean level of IL-10 protein in PBMC of CHF patients with function classes I, II, III and IV were 292.9 ± 193.4, 613.5 ± 166.1, 399.4 ± 283, and 314.2 ± 250.2, respectively. However, the difference between groups was not significant ($p > 0.05$) (Tab. 3).

Discussion

Inflammation plays a significant contributory role in the pathogenesis of chronic heart failure (CHF). Several cell types and cytokines are involved in orchestrating this complex disease. Numerous studies have shown that T cells are involved in cardiac remodeling, and significantly alter the cardiac pathophysiology. Two distinct subsets of T helper ($CD4^+$), Treg and Th17 cells have crucial regulatory roles in cardiac function. Tregs have been identified as suppressors of diverse immune responses and inflammation, whereas Th17 promote inflammation by IL-17 production. Based on the cytokine-regulated balance of these two "master regulators", Foxp3 and RORγt, Treg and Th17 would be differentiated, respectively.

A reduced number of circulating Treg with compromised function and less Foxp3 expression in PBMC in CHF patients have been previously reported (18). Moreover, it has been shown that Treg deficiency correlates with the severity of CHF and is reduced in both IHF and NIHF, which seemed to be independent of etiology (18).

The results of this study could not confirm any significant difference for Foxp3 between CHF and control groups. The level of Foxp3 did not show any correlation with CHF severity, but it appeared to be correlated with the cause of CHF as the level of Foxp3 was significantly lower in IHF vs NIHF patients.

Several previous studies have demonstrated the dysregulation of Foxp3 expression in numerous inflammatory diseases. A reduction in disease severity in models of Diabetes Mellitus, Multiple Sclerosis, asthma and Inflammatory Bowel Disease by Foxp3 administration has been reported too (19, 20).

Since the balance between Foxp3 and RORγt has been demonstrated previously, we compared RORγt as well. Our result did not find any significant difference for RORγt between CHF patients and the normal group. In addition, there was neither significant correlation between RORγt level and CHF etiology, nor with CHF severity. The role of RORγt as potential therapeutic target for controlling inflammation in autoimmune diseases has been discussed by Huang et al (21).

In this regard, Hu et al identified the cardiac glycoside digoxin inhibit RORγt transcriptional activity and attenuate inflammatory lymphocyte function and autoimmune disease (22).

Targeting RORγt to therapeutically suppress inflammatory T cell function in numerous autoimmune disorders including ar-

thritis rheumatoid, psoriasis, autoimmune hepatitis and spondyloarthropathy has been reported previously (23-26). The role of Th17 and Treg in acute coronary syndrome (ACS) and myocardial infarction (MI) has been reported too. In this regard Cheng et al have showed a significant increase in peripheral Th17 number, Th17 related cytokines and RORγt levels and a decrease in Treg number and Foxp3 levels in ACS patients (27). In addition, Barry et al revealed enhanced expression of IL-17 T cell, IL17 cytokines and RORγt following ischemic reperfusion injury in MI models of rats (28).

Studies regarding CHF are sparse and lead to controversial findings (29, 30).

However, Li et al have stated a promising therapeutic approach by harmonizing the Th17/Treg balance in patients with CHF (29). The significant difference in circulating Th17, IL17and RORγt between CHF patients and normal individuals was not confirmed by another study (30).

Since cytokines communicate between each other in a network, the role of anti-inflammatory cytokines is worth noting in inflammatory diseases. IL-10 is a potent anti-inflammatory cytokine which is known to suppress the synthesis of pro-inflammatory cytokines (31).

A previous study in mice models after MI induction and treatment with IL-10 demonstrated the suppressive effect of IL-10 which leads to left ventricular improvement, reduced infarct size, and attenuated infarct wall thinning (32).

Higher level of IL-10 in CHF patients and its correlation with CHF severity has been reported in previous studies (15, 33) which proposed IL-10 as an important inherent component of the cytokine network of CHF.

In the current study, the mean level of IL-10 expression was lower in CHF patients than in control group; the difference, however, was not significant ($p > 0.5$).

In addition, our data could not confirm any correlation between IL-10 level and CHF severity or CHF etiology which may be due to the small sample size in each group of patients.

In summary, this study shows a significant lower level of Foxp3 in IHF vs NIHF. However, we could not confirm any significant changes in RORγt and IL-10 in CHF patient's vs normal subjects. The limitation of the current study was the small size of samples which could have contributed to the failure to reach the statistical significance. Therefore, further research with a larger sample size needs to be conducted to identify more precisely the most important factors in the immunopathogenesis of chronic HF in the pursue of developing more specific immunomodulating agents for this disorder.

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