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Foxp3-Infiltrating Cellular Expression in Rheumatic Mitral Valve Tissue Lesions

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Abstract

Background The Foxp3 gene is exclusive. It found in nTregs and correlates with the suppressive activity of these cells.

Objective To detect Foxp3 expression in infiltrating cells in the rheumatic mitral valve tissue lesions and its correlation with the extent of histopathological abnormalities when considered naturally occurring CD4+CD25+ regulatory T cells (nTregs) as the main expressers for this protein.

Methods Rheumatic mitral valve surgical fragments were taken from a total of 48 Iraqi patients with chronic rheumatic heart disease underwent mitral valve replacement in Ibn Al-Bitar Hospital for Cardiac Surgery-Iraq-Bagdad from October 2006-September 2007. Formalin-fixed paraffin-embedded mitral valve tissue sections were prepared. Foxp3-expressing cells were detected by using immunohistochemical staining technique, and histopathological picture was visualized by using hematoxylin and eosin staining.

Results Our results showed that there were no significant association between Foxp3 expression and the history of acute rheumatic fever (negative or positive), and/or the frequency of attacks (single or multiple) among all groups under study ($p > 0.05$). Also, we found a significant negative correlation between the percentage of Foxp3 strong positive cells and the extent of histopathological abnormalities.

Conclusions There was a negative correlation between Foxp3 strong positive expression and the extent of histopathological abnormalities which reinforce the immunosuppressive role of nTregs against the inflammatory and autoimmune reactions in chronic rheumatic heart disease.

Key words Foxp3, CD4+CD25+ regulatory T cells, mitral valve

Introduction

The Foxp3 gene encodes the protein Scurfin, a member of the forkhead \winged-helix family of transcriptional regulators and is highly conserved in humans [(1)]. It is exclusively found in naturally occurring CD4+CD25+ regulatory T cells (nTregs) and correlates with the suppressive activity of these cells. In mice, Foxp3 is almost exclusively expressed intracellulary in nTregs, whereas in humans, its expression is also detected in extrathymically generated regulatory T cells [(2)].

Foxp3 has been shown to be important for nTregs function [(3)]. There is also evidence suggest that Foxp3 may be important for the induction of nTregs [(4)], in that the introduction of the Foxp3 gene into CD4+CD25- cells resulted in the generation of an anergic\suppressor phenotype similar to regulatory T cells [(5)] .These cells were able to suppress T cell activation in vitro.

Unlike CD25, cytotoxic T lymphocyte associated antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor-related gene

(GITR), and lymphocyte activation gene-3 (LAG-3) markers proposed to define the Treg cell population, Foxp3 is not upregulated in recently activated CD4+CD25- T cells. Only a subset of the Foxp3-expressing cells upregulated CD25 expression and acquired suppressor function after 2 weeks in vivo. More aggressive autoimmune syndrome resulting from genetic deficiency in Foxp3 as compared to that resulting from CD25+ T cell depletion [(6)]. Analogous mutation of human Foxp3 was found to be responsible for immunodysregulation, polyendopathy, enteropathy, X-linked diseases (IPEX) [(1-8)]. In both mouse and human, the Foxp3 mutation is responsible for the most aggressive autoimmune diseases that resulted in early lethality. More importantly, targeted mutation of Foxp3 in hematopoietic cells is both necessary and sufficient to ablate nTregs development. Analysis studies of the immunological basis of autoimmune diseases associated with Foxp3 mutation observed a very substantial reduction in thymic cellularity. The reduction was caused by a decrease in the proliferation of immature thymocytes that lake both CD4 and CD8 co-receptors, thus autoimmune diseases associated with Foxp3 mutation require both T cell-intrinsic defects that result in defective development of regulatory T cells and T cell-extrinsic defects in thymocyte production [(9)]. Thus, the aim of this study was to detect Foxp3 expression in infiltrating cells in the rheumatic mitral valve tissue lesions and its correlation with the extent of histopathological abnormalities.

Methods

This study was conducted from October 2006 to September 2007. Rheumatic mitral valve surgical fragments were taken from 48 patients with chronic rheumatic heart disease underwent mitral valve replacement surgery in Ibn Al-Bitar Hospital for Cardiac Surgery.

All patients were divided according to the positive or negative history of rheumatic fever (PHORF and NHORF), PHORF patients were subdivided according to the frequency of rheumatic fever, and according to the period of medical treatment into single attack under continuous medication (SA^{UCM}), single attack without continuous medication (SA^{WCM}), high risk under continuous medication (HR^{UCM}), and high risk without continuous medication (HR^{WCM}). Negative controls for mitral valve tissue samples were taken from 20 cadavers, their cause of death was not related to the acute or chronic rheumatic heart disease, infective endocarditis or any other heart disease, and their age and sex were matched with chronic rheumatic heart disease patients. Paraffin embedded mitral valve tissue sections (5µm thickness) were prepared using positive charge slides (Fisher Scientific, USA).

Hematoxylin and eosin staining was performed on mitral tissue sections for each patient. Mouse anti- Human Foxp3 protein (Serotic, UK) were used to detect Foxp3 expression in mitral valve infiltrating mononuclear cells using immunohistochemical staining technique.

Statistical Analysis

Foxp3 signals were evaluated by counting the number of positive cells which adhere to the valvular endothelium and the total number of infiltrating cells in 5 to 50 microscopic fields to measuring the percentage of positive cells which was calculated according to the following equilibrium:

The percentage of positive cells = the number of positive cells / the number of total cells X 100

The immunohistochemistry signals of Foxp3 expression on infiltrating cells demonstrating on the mitral valve tissue sections were considered +, (< 10%); ++, (10 to 50%); and +++, (> 50%) as positive cells [(10)]. All statistical analysis was performed with the statistical package for social sciences (SPSS 10.01) and also Excell 2003. A p value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Hematoxylin and eosin stained tissue sections of rheumatic mitral stenosis appeared different

degrees of fibrosis, inflammatory cellular infiltrates, neovascularization and mineralization (Figure 1).

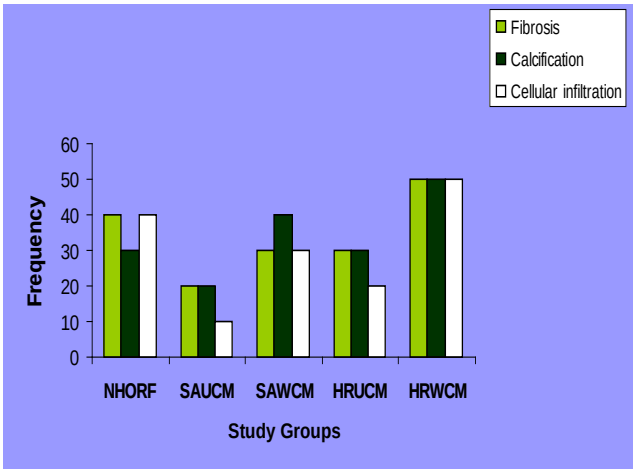

Figure 1: The degree of fibrosis, calcification, and cellular infiltration among different study groups

The clinical features were graduated in severity among all patients due to the continuous inflammatory processes against the heart, as a result, highly significant differences were shown in histopathological features among different study groups when compare between them. In general high risk patients were display very large degree of histopathological abnormalities (HPA) (fibrosis, calcification, and cellular infiltration) which reflects the more severity of disease than other groups, and there was a highly significant difference between the degree of CI in the patients of HR and SA- groups ($p < 0.01$). Medical care groups (SA^{UCM} and HRUCM) were exhibited no or very low-grade of the inflammatory response and subsequently low heart lesion, fibrosis and calcification when compared with patients of intermittent medical therapy (SA^{WCM} and HR^{WCM}).

There was no significant difference in the severity of disease between negative history and SA^{WCM} groups except the increasing in the cellular infiltration in the negative history population with subclinical symptoms which leads to more heart damage. Immunohistochemical staining for Foxp3 was detected as a brown color precipitated in the nuclei of mitral valve infiltrating mononuclear cells (Figure 2), Foxp3 positive cells percentage is shown in (Table 1).

Table 1: Mean percentage of Foxp3 positive cells from the total number of mitral infiltrating mononuclear cells among study population groups

Group type		Patients		ICsC	Foxp3 positive cells		Total Foxp3 value
		No.	(%)		No.	Mean%± SD	
NHORF		14	20.59	33.36	16.71	48.8±12.675	++
PHORF	SA^{UCM}	5	7.35	25.6	6.6	25.48±9.068	++
	SA^{WCM}	18	26.47	36.89	23.67	61.47±13.127	+++
	HR^{UCM}	4	5.88	27.25	9.5	31.32±20.966	+++
	HR^{WCM}	7	10.29	51.71	41.71	80.34±4.563	+++

$\chi^2 = 8.547$, $p > 0.05$; SD = Slandered Deviation; Total value = + (< 10) %, ++ (10-50) %, +++ (≥ 50) %. ICsC = Infiltrating cells count; NHORF = negative history of rheumatic fever; PHORF = positive history of rheumatic fever; SA^{UCM} =single attack under continuous medication; SA^{WCM} =single attack without continuous medication; HR^{UCM} = high risk under continuous medication; HR^{WCM} =high risk without continuous medication.

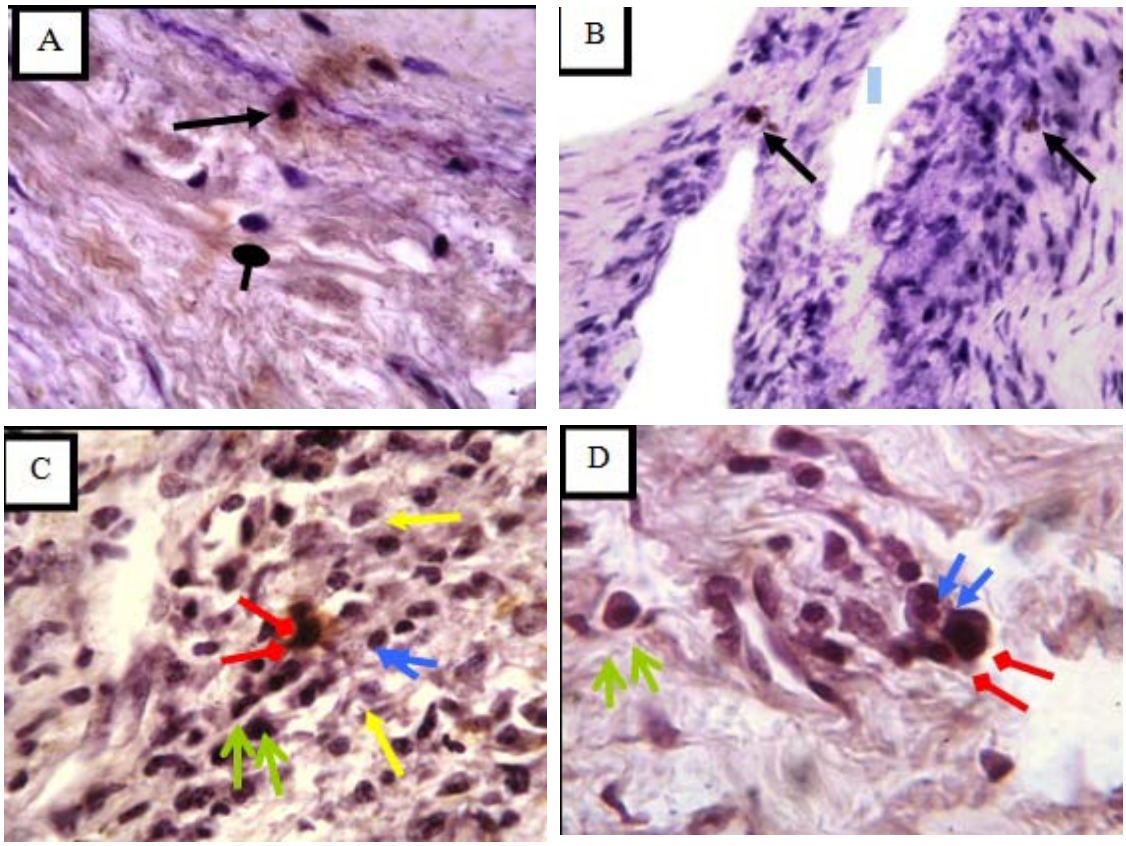

Figure 2: Immunohistochemical staining for human Foxp3 transcriptional factor in the rheumatic mitral tissue sections. (A&B) show Foxp3 positive cells (10-50%) (++). Pin point long arrows indicate Foxp3 positive cells whereas circled short arrows indicate negative cells. (C&D), show Foxp3 positive cells (> 50%) (+++) Foxp3 positive cells infiltrates the myocardium and illustrate various degrees of Foxp3 immunoreactivity, weak positive signals are indicated by blue arrows, positive signals are indicated by green arrows, red arrow indicate strong Foxp3 positive cells staining, and yellow arrows indicate negative Foxp3 signals. Microscopic magnification power: X400 for B; X1000 for A, C, and D.

In general, Foxp3 expression was high with some exceptions. When very high Foxp3 positive cells was shown in the mitral valve of SA^{WCM} (61.47%), and HR^{WCM} patients (80.34%), very low number of Foxp3 positive cells was infiltrate the mitral valve of patients under continuous medication (SA^{UCM} 25.48%, HR^{UCM} 31.32%) but, intermediate to high Foxp3 expression level was found in the patients with negative history (48.8%). Chi-analysis (Table 1) showed no significant statistically association between Foxp3 expression and the history of ARF (negative or positive), and/or the frequency of attacks (single or multiple) among all studied groups (χ^2 = 8.547, p > 0.05). However, HR^{UCM} group included 2 patients who displayed high Foxp3 positive cells, the first patient recorded 51.51% positive Foxp3 from the total number of intralesional mitral valve infiltrating mononuclear cells, and 46.87% of Foxp3 positive cells were found in the second patient. Negative Foxp3 expression was not recorded in any of chronic rheumatic heart disease patients. We now known that the presence of nTregs at the inflammatory sites will reverse the aggressive activity of CD4+ T cells by a certain pathway of suppression, but according to these results, there was no correlation between the high predominance of Foxp3 positive cells in all groups and the extent of histopathological abnormalities. However, non of cases with lower Foxp3 expression

showed severe histopathological abnormalities and not all cases with lower histopathological abnormalities had large number of Foxp3 positive cells except one SA^{UCM} patient who exhibited (30.43%) positive Foxp3 expression and at the same time showed low fibrosis and calcification with very low number of mitral valve infiltrating mononuclear cells (23 cell/field). According to the degree of Foxp3 immunostaining (saturation of pigment), we were found different immunostained intensities are recorded as negative, weak positive, positive, and strong positive (Table 2).

Table 2: The degree of the Foxp3 immunostaining signals in mitral infilterating mononuclear cells in different study groups

Group type		MeanFoxp3 positive cells (No.)	Signal immunostaining				Total
			Negative	Weak +ve	Positive	Strong +ve	
NHORF		17	0 (0.00%)	5(29.41%)	8(47.06%)	4(23.53%)	17(100%)
PHORF	SA^{UCM}	7	0 (0.00%)	0 (0.00%)	4(57.14%)	3(42.86%)	7 (100%)
	SA^{WCM}	24	0 (0.00%)	6 (25%)	16(66.67%)	2 (8.33%)	24(100%)
	HR^{UCM}	10	0 (0.00%)	3 (30%)	4 (40%)	3 (30%)	10(100%)
	HR^{WCM}	42	0 (0.00%)	7(16.67%)	34(80.95%)	1(2.38%)	42(100%)
Control		0	20(100%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	20(100%)

NHORF = negative history of rheumatic fever; PHORF = positive history of rheumatic fever; SA^{UCM} =single attack under continuous medication; SA^{WCM} =single attack without continuous medication; HR^{UCM} = high risk under continuous medication; HR^{WCM} =high risk without continuous medication.

Discussion

Foxp3 which was known as the master regulator of CD4+CD25+ nTreg cells and considered the more specific marker for the identification of these cells. In this study we found that this transcriptional factor was highly expressed by the mitral valve infiltrating mononuclear cells and (48.8%) from the total infiltrating mononuclear cells were positive for Foxp3 protein in the negative history patients, (25.48%) in SA^{UCM}, (61.45%) in SA^{WCM}, (31.32%) in HR^{UCM}, and (80.34%) in HR^{WCM} groups. These values give completely different picture than the expected one when considered that the CD4+CD25+ nTreg cells (which were found in 5-10% of the normal the peripheral blood) are the target of Foxp3 immunoreactivity. Early reports suggested that proportion of human CD4+ T cells always express Foxp3+ when activated, and that these cells then become phenotypically and functionally indistinguishable from nTregs[11]. In human, Foxp3 expression is readily induced in majority of both human CD4+CD25+Foxp3- and CD8+CD25+Foxp3- T cells by the activation via the T cell receptor (TCR), whereas Foxp3 expression is not induced in mouse CD25-T cells under similar activating conditions. The expression of Foxp3 after TCR stimulation of human CD25- T cells can approach that of the resting CD4+CD25+Foxp3+ population. These studies may explain the high predominance of Foxp3 expression by infiltrating mononuclear cells in the rheumatic mitral valve lesions since of course, all CD4+ T cells which infiltrate the autoimmue heart lesion are activated cells, but now there is evidence that Foxp3+ T effector cells does not suppress the proliferation or cytokine production of Foxp3- CD4+ T cells or

other immune cells which usually suppressed by naturally occurring Treg cells [(12)]. In other words, the induced Foxp3+ population is neither anergic nor suppressive in vitro, and the expression of Foxp3 appears to be transient, declining to baseline amounts with prolonged culture. Thus, the high percentage of Foxp3 positive cells may be as a result from the presence of both CD4+CD25+ nTregs and activated effector CD4+ T cells. Therefore, in this case, false positive result was obtained here may explain why no significant correlation was found between the positive expression of Foxp3 and the extent of histopathological abnormalities. Also this study found that the lower severity of the histopathological picture was highly significant associated with the high numbers of strong positive Foxp3 expression, and these results reinforce the immunosuppressive function of CD4+CD25+ regulatory T cells. Whereas, the mean percentage of positive /weak-positive Foxp3 expression was found to be positively correlated with the extent degree of calcification ($p < 0.01$), fibrosis and cellular infiltration ($p > 0.05$).

Our findings revealed that Foxp3-CD4+CD25+ regulatory T cells play an important role in controlling the autoimmune process against the heart in rheumatic heart disease, and this may open new future ways for using nTreg cells in immunotherapy.

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