Inflammatory Cytokines, Apoptotic, Tissue Injury and Remodeling Biomarkers in Children with Congenital Heart Disease

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Abstract The present study aims to evaluate specific biomarkers involved in congenital heart disease (CHD), and whether there is a significant differences between the levels of these biomarkers in the cyanotic CHD (CCHD) and acyanotic CHD (ACHD). We prospectively measured tumor necrosis factor (TNF- α), interleukin-6 (IL-6), C-reactive protein (CRP), vasoendothelial growth factor (VEGF), troponin T, creatin kinase MB (CKMB), and Caspase 3 levels in 120 consecutive children with CHD (60 cyanotic and 60 a cyanotic with age 1:4 years), and 30 healthy control children. Significant elevated levels of inflammatory markers; TNF- α , IL-6 and CRP was detected in CHD, with percentage increase in cyanotic than a cyanotic subjects as compared to the normal one. Apoptotic biomarker; caspase 3 showed also significant increases in CCHD than ACHD. In addition, tissue injury mechanisms included troponin T and CKMB, exhibited significant increase in cyanotic than a cyanotic CHD. The present results demonstrate also, significant enhancement in remodeling process (VEGF), in cyanotic than a cyanotic patients. Thus, it could be concluded that, the children with CCHD were shown to have elevated levels of inflammatory cytokines, caspase 3, troponin T, and CKMB as these biomarkers may implicated in cardiac functional status.

Keywords Cyanotic heart disease · Acyanotic heart disease · TNF- α · IL-6 · Caspase 3 · VEGF

Introduction

It is well known that, CCHD in children commonly causes growth retardation. Patients with CHD are prone to malnutrition for several reasons including decreased energy intake, increased energy requirements, or both [1].

It has been demonstrated that adults with CHD with a broad spectrum of diagnoses have clinical features and a pattern of neurohormonal activation characteristically found in chronic heart failure (CHF). It is not known whether the similarities between adult congenital heart disease and CHF extend to the cytokine system [1]. There is a single report describing elevated TNF- α level in a small pediatric cohort with atrial septal defects [2]. There have been no reports investigating the state of the cytokine system activity in infants or adults with CHD. Establishing a potential between immune activation and the pathogenesis of CHD may have important clinical and therapeutic implications. Furthermore, patients with the most advanced disease have the greatest degree of inflammatory cytokine activation. There was a significant correlation between serum levels of tumor necrosis factor receptor-1 (sTNFR-1), and the degree of impairment of systemic ventricular function. It was found that, the patients with advanced CHF had increased concentrations of circulating TNF- α , especially those who were cachectic and the typical constellation of microspherocytic and hypochromatic red blood cells is often absent in CCHD [3].

Recent study documented elevated expression of myocardial VEGF and the increase in the number of microvessels in the myocardium of CCHD diseases [4].

Cardiac troponin T, is an established specific marker of myocardial damage in adult patients [5]. The diagnostic value of troponin T and CKMB in myocardial damage of pediatric patients was previously studied [5]. This study will add evidence both supportive and otherwise, that biomarkers are in

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fact useful in pediatric heart failure. In so doing we will examine the evidence for the use of specific biomarkers likely tropinin T, CKMB, caspase 3, interleukins-6, TNF- α and CRP to be implicated in tissue injury, remodeling and inflammation.

Materials and Subjects

Patients

60 patients with CCHD (26 boys, 34 girls), and 60 patients with ACHD (35 boys, 25 girls), in addition to 30 normal control (30 males and 30 females), with ages 1–4 years as they were admitted to the Department of Pediatric Cardiology in National Heart Institute (Imbaba, Egypt), were included in this study. None of the patients had associated abnormalities or pulmonary hypertension. All patients' cardiac diagnoses were made on the basis of clinical and laboratory examinations including telecardiography, electrocardiography and echocardiography. Diagnoses were confirmed by cardiac catheterisation in cyanotic group. The specific cardiac lesions of patients are listed in Table 1. None of the patients included in this study had acute illness at the time of the study. A total of 120 children with CHD who met the inclusion criteria constituted the study population. The Informed consents were taken from the parents of our studied groups according to guideline of the Medical Ethical Committee of National Research Centre, Giza, Egypt. All the studied groups were subjected to full history report including personal history, complete present history, family history, social history and past history.

Blood Samples

Fasting blood samples were obtained and collected from the antecubital vein using a lightly fitting tourniquet. Three

ml blood was drawn from each subject after fasting for 12 h. Sample was taken in a sterile tube; sera were obtained immediately after clotting of samples, collected in sterile tubes and stored at -80°C until used. The first sample was drawn into a syringe containing 0.25 ml of 3.8 % sodium citrate to measure CRP activity.

Serum Biochemical Analyses

Determination of Troponin T, TNF- α , IL-6, CK-MB

Serum troponin T and CK-MB, levels were measured using commercially available ELISA assays (DuoSet kits, R&D Systems; Minneapolis, MN, USA). The results are shown as pg/ml and U/l respectively.

Determination of CRP Level

CRP was measured using latex-enhanced immunonephelometry on a Behring Nephelometer (Dade Behring). The lower detection limit of the assay was 0.15 mg/l [11].

Determination of VEGF Level

The level of VEGF in serum was determined at 492 nm by quantitative colorimetric sandwich enzyme linked immunosorbent assay (ELISA systems, UK) in accordance with the manufacturer's instructions. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer.

Caspase 3 Activity Assay

Caspase-3 was measured using fluorescence Cytoor 2300 at excitation 380 nm, emission 460 and at 60 min [7].

Statistical Analysis

The results are expressed as mean $\pm$ SD. Data of the three groups were analyzed using Co-state computer program, where unshared letter is significant at $p \leq 0.05$. Correlations between tissue injury markers and other serum parameters in the CCHD and ACHD groups were analyzed using Pearson's correlation analysis.

Results

Significant increase was detected in TNF- α , IL-6 and CRP in CCHD and ACHD with percentage increase of 3.69, 54.40 and 115.48 %, respectively for cyanotic children. While acyanotic subjects exhibited percentage increase reached to 11.15, 54.16 and 61.26 %, respectively. In addition, the

Table 1 Diagnosis of the patients

Diagnosis	No.	Age (years)
Cyanotic patients	9	
Tetralogy of fallot	7	1:4
Truncus arteriosus 3	7	
Double outlet right	7	
Ventricle 3	7	
Pulmonary atresia 2	6	
Pulmonary stenosis 2	6	
Tricuspid atresia 2	6	
Eisenmenger syndrome	5	
Acyanotic patients		
Ventricular septal defect	20	1:4
Atrial septal defect 4	12	
Patent ductus arteriosus	28	

remodeling marker; VEGF showed significant increase in both cyanotic and acyanotic congenital heart with percentage increase 14.86 and 7.71 %, respectively. Furthermore, the markers of tissue injury; troponin T and CKMB declared significant increase in both cases of CHD with percentages increase 63.85 and 53.85 %, respectively for ACHD. While, the elevated levels reached to 66.15 and 56.30 %, respectively in cyanotic patients (Table 2; Fig. 1). Significant correlation was noticed between tissue injury biomarkers and other biomarkers representing inflammatory cytokines, apoptosis and remodeling (Table 3).

Discussion

Markers of Inflammation; IL-6, CRP and TNF- α

The present results reveal, significant increase in IL-6, CRP, TNF- α in cyanotic than acyanotic CHD as compared to normal subjects. In a cross-sectional assessment of children with symptomatic heart failure, it was found that, the elevation of cytokines reacting with both the clinical severity of the disease and the degree of remodeling [6]. The significant elevation in CRP is in a good agreement with Kantor and Rusconi [7], who found that CRP is associated with symptom severity, and able to discriminate between clinical severity groups.

Moreover, transmembrane-protein(TM-protein) and C-protein S pathway are a typical endothelial anticoagulant system in contrast to the pro-coagulant activity of P-selectin, the down-regulation of TM has been reported in adult patients with pulmonary hypertension [8]. In fact, Cadroy et al. [9] reported that the expression of TM, CRP and the amount of fibrin deposition on endothelium exposed to high-shear stress was comparable with those of endothelium treated with anti-TM antibody, indicating the role of chronic high-shear stress in the down-regulation of TM. It was found also that, protein C activity and plasma

Fig. 1 % Change of different biomarkers in congenital heart disease

level of protein S were decreased in CCHD group, predisposing toward thrombosis in microcirculation [10].

The concept that several tiers of inflammatory activation may contribute to remodeling is suggested by the experience with the angiotensin-receptor 1 antagonist, candesartan, which was reported to decrease CRP levels but not interleukin levels in moderately severe heart failure [11]. The present results clearly demonstrate that IL-6 was significantly increased in both CCHD and ACHD with percentage increase 54.40 and 54.166 %, respectively. The evidence to support the hypothesis that immune activation may be present in patients with CHD comes from the report of Lequier et al. [12]. In their study, it was noted that 40 %, of infants with a range of congenital heart defects were significantly endotoxemic, and this was found to relate adversely to clinical outcome. Bacterial endotoxin, a potent stimulus for inflammatory cytokine especially IL-6 production, has been proposed to play an important role in the pathogenesis of chronic heart failure (CHF) [13]. In concomitant with the present results, Sharma et al. [3], found that cyanotic patients had significantly increased levels of inflammatory cytokines compared with acyanotic patients, and noticed that, there was also a trend toward higher endotoxin levels in the former group. These results suggested that, cyanosis itself may represent an important stimulus for immune activation in adults with CHD. This is consistent with previous work, which has shown that

Table 2 Serum biomarker levels in control, cyanotic and acyanotic CHD

Parameters group	Control	Acyanotics	Cyanotic
TNF- α	148.39 $\pm$ 2.5521 ^a	164.94 $\pm$ 21.982 ^b	183.55 $\pm$ 3.71 ^c
CASP3	83.79 $\pm$ 2.5837 ^a	301.03 $\pm$ 13.739 ^b	340.33 $\pm$ 14.50 ^c
CKMB	32.15 $\pm$ 1.3719 ^a	43.83 $\pm$ 2.2145 ^b	49.46 $\pm$ 1.09 ^c
TROPONIN-T	21.50 $\pm$ 1.2570 ^a	35.73 $\pm$ 5.508 ^b	39.54 $\pm$ 0.67 ^c
IL-6	10.48 $\pm$ 0.4481 ^a	16.15 $\pm$ 0.769 ^b	20.33 $\pm$ 1.52 ^c
VEGF	167.73 $\pm$ 2.5334 ^a	180.66 $\pm$ 4.0415 ^b	192.66 $\pm$ 5.50 ^c
CRP	7.27 $\pm$ 0.2542 ^a	11.73 $\pm$ 0.2053 ^b	15.66 $\pm$ 0.57 ^c

Data are expressed as mean $\pm$ SD of 30 control and 120 patients with cyanotic and acyanotic CHD. TNF- α , TROPONIN-T, IL-6 and VEGF are expressed in pg/ml, CASP3 and CRP are expressed in ng/ml while CKMB is expressed in U/l. Statistical analysis is carried out using Co-state computer program, where unshared letter is significant at $p \leq 0.05$

Table 3 Person's correlations between cytokines, tissue injury and remodeling biomarkers

Parameters	TNF	IL-6	CRP	Caspase3	CKMB	Troponin	VEGF
TNF	1	0.935**	0.985**	0.945**	0.897**	0.924**	0.927**
IL-6		1	0.975**	0.946**	0.955**	0.971**	0.912**
CRP			1	0.980**	0.975**	0.922**	0.937**
Caspase3				1	0.0.897**	0.946**	0.903**
CKMB					1	0.985**	0.924**
Troponin						1	0.879**
VEGF							1

** Correlation is significant at $p \leq 0.01$ level (2-tailed)

hypoxia can lead to inflammatory cytokine release in patients with heart failure [14]. Our findings suggested that, CHD in children has an inflammatory component, and cyanosis is a potential stimulus for immune activation.

Nitric oxide bioavailability is increased in CCHD because increased endothelial shear stress of erythrocytosis is a major factor in nitric oxide elaboration and nitric oxide synthase (eNOS) gene expression [15]. In addition, red blood cells are considered as nitric oxide reservoirs causing elevation in red cell mass [16]. In CHD extramural coronaries dilate in response to nitric oxide and prostaglandins elaborated in response to endothelial shear stress induced by the viscous erythrocytosis perfusate, but dilatation often exceeds the anticipated vasodilator response [17].

It is believed that, there is signal transduction process acts as an important mechanism for TNF- α induced activation of caspases-3, thereby, initiating apoptosis and therefore cell death. The existence of functional of which contain a death domain (DD) in their tumor necrosis factor receptor-1 (TNFR-1), in the human heart patients with congestive heart failure have been reported [18]. Apoptosis in the cardiovascular system has been demonstrated, however, evidence of apoptosis has been reported from diverse aspects of cardiovascular medicine, ranging from CHF to conduction system defects to coronary atherosclerosis [19].

The occurrence of apoptosis in patients with CHF suggests that apoptosis may play a role in the progression of the disease and the chronic remodeling of the myocardium that occurs in heart failure [20].

Perloff et al. [21], imply remodeling of the intra-myocardial coronary microcirculation, perhaps vasculogenesis in response to hypoxemic stimulation induced by VEGF from myocardial smooth muscle cells and up-regulation of VEGF receptor-1 in heart endothelial cells.

The present results show that, serum level of VEGF was significantly elevated in CHD with percentage increase in cyanotic (14.86 %) than acyanotic one (7.71), as compared to normal control children. The elevated serum VEGF was

found to be correlated with the degree of cyanosis in patients with CCHD disease, suggesting that, the increase in VEGF could contribute to active neovascularization in patients with CCHD [22]. Our results showed increased serum VEGF actually contributes to endothelial cell and supported this hypothesis. There are multiple growth factors involved in the regulation of blood vessel formation including vasculogenesis, angiogenic remodeling, vessel stabilization and maturation. In these steps, interactions of these molecules are carefully regulated to form a functioning vascular network [22].

The increased level of VEGF and HGF may be, referred to as angiogenic growth factors, stimulate the development of collateral arteries of ischemic heart disease [23].

The present results declare, significant increase in the troponin T in both cases of CHD with higher percentage in cyanotic (66.15 %), than acyanotic (63.85 %), subjects as compared to normal control. Kantor and Rusconi [7], found that, the new onset of heart failure could be attributed to the high level of troponin T and CRP in patients with stable coronary artery disease and preserved systolic function.

It was demonstrated that, in healthy adults virtually no cardiac troponin T is demonstrable, so that every rise of the level of the heart-specific troponin T in the blood means that there is myocardial damage [24, 25]. According to the guide lines of the National Academy of Clinical Biochemistry and the International Federation of Clinical Chemistry, cardiac troponin T is considered as one of the new 'gold markers' of ischemic myocardial injury. The elevation of cardiac troponin T is greater in ischemic injury (11-fold) but it is less in non-ischemic injury (fivefold) [25].

The present study, shows that serum level of CKMB was significantly elevated in children with CHD, as compared to normal control one with percentage of 63.85 and 53.85 %, respectively for cyanotic and acyanotic heart. It is of note that the mean basal CKMB levels were abnormal in pulmonary hypertensive, younger patients and in patients medicated for CHF [25]. Recent clinical studies suggested

an improved cardiac specificity for troponin T and I compared with CKMB for detection of myocardial injury in the presence of skeletal muscle injury [25]. Thus, it could be concluded that, congenital heart disease in children has an inflammatory component, and cyanosis is a potential stimulus for immune activation, apoptosis, tissue injury and remodeling. In addition, further investigation is required to improve our understanding of the late pathogenic mechanisms involved in patients with CHD, which may lead to novel anti-inflammatory therapies.

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