Comparative study of the roles of cytokines and apoptosis in dilated and hypertrophic cardiomyopathies

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ABSTRACT. Aims. In order to gain more insight into the pathogenesis of dilated and hypertrophic cardiomyopathies (DCM and HCM, respectively), we investigated the roles of certain cytokines that regulate apoptosis. Methods and results. ELISA tests, performed to determine the plasma concentrations of tumour necrosis factor-α (TNF-α), the soluble Fas (sFas), interleukin-6 (IL-6) and the soluble IL-6 receptor (sIL-6R), revealed that DCM patients exhibit elevated concentrations of TNF-α, sFas, IL-6 and sIL-6R, while HCM patients have only high IL-6 and sIL-6R levels as compared with healthy individuals. Western blot analysis of the levels of TNF-α, IL-6, Bcl-2 and Bax proteins in myocardium samples demonstrated that DCM patients express increased levels of TNF-α, IL-6 and Bax, whereas HCM heart lysates display only elevated levels of Bcl-2. Annexin V binding assay of TNF-α -treated H9C2 cells indicated that the in vitro cytotoxicity of this cytokine involves apoptosis and necrosis. Conclusion. In accord with previous observations, our data indicate a strong activation of the pro-apoptotic TNF and Fas pathways in DCM patients, and an anti-apoptotic shift in HCM patients. These findings have a bearing on the pathogenesis of cardiomyopathies, since apoptosis may account for certain dysfunctions observed in DCM, while IL-6 may elicit the hypertrophy characteristic of HCM.

Keywords: DCM, HCM, TNF-α, Fas, IL-6, apoptosis

Cardiomyopathies are diseases of the heart muscle leading to cardiac dysfunction and heart failure [1]. Primary cardiomyopathies are due to inherited gene defects, while the secondary forms can be caused by a great variety of noxious conditions, including ischaemia, inflammation, infections and toxic compounds [1, 2]. Cardiomyopathies have been classified into four groups: (i) dilated cardiomyopathy (DCM), (ii) hypertrophic cardiomyopathy (HCM), (iii) arrhythmogenic right ventricular dysplasia and (iv) restrictive cardiomyopathy. DCM is characterized by acontractile dysfunction and dilatation of the ventricles [1]. HCM manifests as hypertrophy of the ventricles, with histological features of myocyte hypertrophy, myofibrillar disarray and interstitial fibrosis [1]. The chronic biomechanical stress, contractile dysfunction and hypoxia lead to progressive deterioration of ventricular function and evoke the activation of a complex cascade of compensatory mechanisms to maintain cardiac function [3].

Previous studies have revealed that proinflammatory cytokines are important contributors to the cardiac dysfunction in numerous cardiovascular disorders [reviewed in 4, 5]. Recent observations have indicated that the pathogenetic role of cytokines in heart diseases is connected with their ability to modulate (i.e. induce or inhibit) the process of apoptosis of cardiac myocytes [6]. Tumour necrosis factor-α (TNF-α) exerts a cardiotoxic effect through different mechanisms. It depresses the contractile functions, promotes left ventricular remodelling, diminishes β-adrenergic inotropic responsiveness, and activates both anti-apoptotic and pro-apoptotic pathways in the myocardium [7-11]. Interaction between the Fas ligand (FasL) and Fas may also mediate the apoptotic demise of cardiac myocytes under certain pathophysiological conditions, while the normal myocardium and cardiomyocyte cultures exhibit some resistance to Fas-mediated cytotoxicity [12-14]. The TNF and Fas pathways of apoptosis have already been implicated in the pathogenesis of myocardial infarction, ischaemia-reperfusion injury, cardiomyopathies and congestive heart failure [6, 10, 12-16]. IL-6 has also been associated with heart diseases, and has been shown to exert a negative inotropic effect, and to regulate hypertrophy and apoptosis [7, 17, 18]. The gp130, Janus kinase (JAK), and signal transducers and activators of the transcription (STAT) signalling pathway, activated by IL-6-related cytokines, has recently been identified as a critical pathway for the survival of cardiac myocytes [19]. Moreover, an impaired activation of the IL-6 pathway has been implicated in the pathogenesis of DCM [19]. Bcl-2 family member proteins, which may be pro-apoptotic or anti-apoptotic, regulate the apoptotic process [20]. Alterations in the expression of the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins have been described in patients with ischaemic heart disease, DCM and heart failure [21]. Furthermore, the transgenic overexpression of Bcl-2 has been shown to be cardioprotective by reducing the rate of
cardiomyocyte apoptosis induced by ischaemia-reperfusion injury [22].

Given the fact that cytokines are produced simultaneously during the course of heart diseases, the overall effect might be due to the complex interactions between pro-apoptotic and anti-apoptotic cytokines, and also soluble cytokine receptors. Multistep control is involved, which might be different in various types of heart failure [23]. The pathogenetic mechanisms of DCM and HCM are not the same, and the exact roles of cytokines in these disorders are not yet fully understood. The aim of our study was therefore to compare the contributions of TNF-α and IL-6 to the pathogenesis of DCM and HCM, and to explore a potential connection between the cytokine pattern and the pro-apoptotic or anti-apoptotic markers in these two different heart diseases.

METHODS

Patients

The details relating to the severity of heart failure of the patients in the different aetiological groups are listed in Table 1. The diagnosis of DCM was based on clinical, serological, echocardiographic and histological data. Patients were examined by right and left heart catheterization. All had an enlarged left ventricle [left ventricular end diastolic diameter ranging from 58 to 87 mm (median 69 mm)], with a reduced ejection fraction of from 14 to 59% (median 29%), according to World Health Organization criteria. Cases of left ventricular dilatation due to coronary heart diseases, valvular dysfunction, hypertension, endocrine disorders or alcohol abuse were excluded. The diagnosis for HCM was based on clinical, electrocardiographic and echocardiographic data. The diagnostic criteria for HCM were defined by a maximum wall thickness > 13 mm on echocardiography or major abnormalities on ECG (abnormal Q waves or marked T wave inversion). The healthy control population comprised 20 blood donors attending the South Hungarian Regional Blood Bank of the National Transfusion Service. All patients gave their written consent before participating. The institutional Ethical Committee approved the study.

<table>
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<tr>
<th>Age (years)</th>
<th>NYHA</th>
<th>LVEF (%)</th>
<th>LVEDD (mm)</th>
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<tbody>
<tr>
<td>DCM (n = 31)</td>
<td>50</td>
<td>4</td>
<td>29</td>
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<tr>
<td>HCM (n = 19)</td>
<td>51</td>
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NYHA = New York Heart Association; LVEF = left ventricular ejection fraction; LVEDD = left ventricular end diastolic diameter.

Enzyme-linked immunosorbent assays (ELISAs) for TNF-α, IL-6, sIL-6R and sFas

Blood samples were taken from the 20 healthy controls [median age 50 years, range 22 to 67 years; 14 men (70%) and 6 women (30%)], 31 DCM patients [median age 50 years, range 24 to 75 years; 24 men (77.4%) and 7 women (22.6%)] and 19 HCM patients [median age 51 years, range 20 to 77 years; 12 men (63.2%) and 7 women (36.8%)] into tubes containing EDTA-2Na. The plasma concentrations of TNF-α, IL-6, sIL-6R and sFas were measured with ELISA kits (BioSource SA, Nivelles, Belgium, for TNF-α; R & D Systems, Inc. Minneapolis, MN, USA, for IL-6, sIL-6R and sFas) according to the manufacturers’ directions. Supplied standards were used to generate the standard curves. The absorbance at 450 nm was converted to international units per millilitre (IU/ml) for TNF-α, to picograms per millilitre (pg/ml) for IL-6, and to nanograms per millilitre (ng/ml) for sIL-6R and for sFas. The detection limit of the assay was 1 IU/ml for TNF-α, 0.2 pg/ml for IL-6, 1.5 pg/ml for sIL-6R, and 20 pg/ml for sFas.

Western blot assays

Endomyocardial biopsies were obtained from DCM patients, myocardial tissue specimens from the hypertrophied septum of patients undergoing septal myectomy for severely symptomatic HCM and control myocardium from victims of sudden accidental death (within 6 h following death). Total protein was solubilized after ultrasonication of the myocardial tissue specimens, with subsequent processing in Laemmli’s sample buffer. The individual protein samples were resolved by SDS-PAGE and electrotransferred onto nitrocellulose filters (Amersham, Buckinghamshire, UK). Preblocked blots were reacted with specific primary antibodies to TNF-α (R & D), IL-6 (R & D), Bax (Santa Cruz Biotechnology Inc., Cambridge, MA, USA) or Bcl-2 (Serotec Ltd, Oxford, UK) for 4 h in PBS containing 0.05% (v/v) Tween 20, 1% (w/v) dried non-fat milk (Difco Laboratories, Detroit, MI, USA), and 1% (w/v) BSA (fraction V; Sigma Chemical Co., St. Louis, MO, USA). Blots were then incubated for 2 h with anti-mouse secondary antibody coupled to peroxidase (Amersham). Filters were washed five times in PBS-Tween for 5 min between each step, and were developed with a chemiluminescence detection system (Amersham).

Detection of apoptosis with Annexin V

Rat cardiomyocyte H9C2 cells were incubated with 100 IU/ml rMu (recombinant murine) TNF-α (Genzyme Corporation, Cambridge, MA, USA) for 4, 8 and 18 h, or were left untreated. Control and TNF-α-treated cells were incubated with fluorescein isothiocyanate (FITC)-labelled Annexin V (BD Biosciences, San Jose, CA, USA) and propidium iodide (PI) (BD Biosciences) for 30 min. Annexin V binds phosphatidylserine, which is translocated to the external cell surface during the initial stages of apoptosis. PI can penetrate the cell membrane during necrosis, when the cell membrane loses its integrity and becomes leaky. Thus, apoptotic cells are stained only with FITC-Annexin V, whereas necrotic cells are stained both with PI and with FITC-Annexin V. The fluorescence intensities of PI and FITC-Annexin V were determined with a FACStar Plus flow cytometer (BD Biosciences).

Statistical analysis

Plasma TNF-α, IL-6, sIL-6R and sFas concentrations are expressed as medians; box plots show the medians, 25th and 75th percentiles, and ranges. Statistical analysis was performed by using the Mann-Whitney test. Differences were considered to be statistically significant at \( P < 0.05 \).
RESULTS

Plasma levels of TNF-α, sFas, IL-6 and sIL-6R in patients with DCM or HCM

Plasma levels of TNF-α, sFas, IL-6 and sIL-6R were measured by ELISA in the 31 DCM patients, the 19 HCM patients and the 20 control subjects.

A significantly higher TNF-α concentration was observed in the DCM patients as compared with the HCM patients and the controls (the median TNF-α concentration in the DCM patients was 75 [range, 22.5 to 133.5] versus 2.0 [range, 0 to 7.5] IU/ml in the HCM patients, \( p < 0.0001 \) and 1.5 [range, 0 to 6] IU/ml in the control subjects, \( p < 0.0001 \) (Figure 1). No significant difference in median TNF-α level was found between the HCM patients and the controls.

The DCM patients exhibited a significantly higher sFas concentration in comparison with the HCM patients and the controls (the median sFas concentration in the DCM patients was 59.5 [range, 6.0 to 141.3] versus 2.8 [range, 0 to 8.0] ng/ml in the HCM patients, \( p < 0.0001 \) and 4.0 [range, 0 to 5.8] ng/ml in the control subjects, \( p < 0.0001 \) (Figure 2). The median plasma level of sFas in the HCM patients was comparable to that measured in the controls.

Both the DCM and the HCM patient groups displayed significantly higher plasma levels of IL-6 as compared with the controls (the median IL-6 concentration in the DCM patients was 195 [range, 10 to 545] pg/ml and in the HCM patients was 250 [range, 90 to 750] pg/ml versus 7.5 [range, 5 to 10] pg/ml in the control subjects, \( p < 0.0001 \), for both) (Figure 3). Furthermore, the assay revealed a significantly higher median IL-6 concentration in the HCM patients than in the DCM patients (\( p < 0.05 \)).

In both the DCM and the HCM patient groups, there were highly increased plasma concentrations of sIL-6R as compared with the controls (the median sIL-6R concentration in the DCM patients was 22.2 [range, 11.0 to 28.0] ng/ml and in the HCM patients was 30.3 [range, 17.3 to 40.0] ng/ml versus 16.2 [range, 6.8 to 17.9] ng/ml in the control subjects, \( p < 0.01 \) and \( p < 0.001 \), respectively) (Figure 4). Moreover, the assay revealed a significantly higher median sIL-6R concentration in the HCM patients than in the DCM patients (\( p < 0.001 \)).

Thus, in the DCM patients, the levels of TNF-α, sFas, IL-6 and sIL-6R were elevated, while only IL-6 and sIL-6R levels were elevated in the HCM patients as compared with the healthy individuals. Moreover, there were significant differences between the two patient groups: the plasma TNF-α and sFas levels were higher in the DCM patients (Figures 1 and 2, \( p < 0.0001 \) for both), while plasma IL-6 and sIL-6R concentrations were higher in the HCM patients (Figures 3 and 4, \( p < 0.05 \) and \( p < 0.001 \), respectively).

Expression of TNF-α, IL-6, Bcl-2 and Bax proteins in the myocardium of patients with DCM or HCM

The steady-state levels of TNF-α, IL-6 and Bcl-2 proteins in myocardium specimens from patients with DCM or
HCM were determined by means of Western blot analysis. Figure 5 presents representative results for two DCM and two HCM samples.

TNF-α could not be detected in the heart lysates of the control subjects and the HCM patients (Figure 5, lanes 5, 6 and 3, 4, respectively). However, the DCM samples contained elevated levels of this cytokine (Figure 5, lanes 1 and 2).

Likewise, IL-6 was not detected in the myocardium of the healthy individuals and the HCM patients (Figure 5, lanes 5, 6 and 3, 4, respectively). In contrast, the DCM specimens revealed highly elevated IL-6 levels (Figure 5, lanes 1 and 2).

The control and DCM heart lysates exhibited low and variable Bcl-2 expression levels (Figure 5, lanes 5, 6 and 1, 2, respectively), whereas levels in the HCM heart lysates were highly elevated (Figure 5, lanes 3 and 4).

The control and HCM heart lysates exhibited low levels of expression of Bax (Figure 5, lanes 5, 6 and 3, 4, respectively), whereas those in the DCM heart lysates were considerably higher (Figure 5, lanes 1 and 2).

Together, these results indicate that the myocardial tissues from the DCM patients express increased levels of TNF-α, IL-6 and Bax, while the HCM heart lysates exhibit only elevated levels of Bcl-2.

**TNF-α induces apoptosis and necrosis in the H9C2 cell line in vitro**

To determine the effect of TNF-α on cultured cardiomyocytes, TNF-α -treated H9C2 cells were analysed by Annexin V binding assay and compared with control cells.

The assay revealed that at the 18-h time point, 1.13% and 9.4% of the total cell population in the control and TNF-α -treated cultures, respectively, were labelled only with FITC-Annexin V (Figure 6A and 6D, respectively). The increased number of Annexin V+ cells in the TNF-α -treated cultures indicates that the in vitro cytotoxicity of this cytokine involves apoptotic mechanisms. Moreover, the TNF-α -treated cultures had a much higher population of double positive cells (35.4%) than that of control cells (0.23%) (Figure 6D and 6A, respectively). The high level of double positive cells after the 18-h treatment might be a consequence of either late apoptotic or necrotic cells. The time course experiments revealed that, at an earlier time point (4 h), 2.34% of the cells were early apoptotic in the lower-right quadrant, but only 1.14% of the cells were double positive in the upper-right quadrant (Figure 6B). After an 8-h treatment with TNF-α, the proportion of early apoptotic cells increased to 4.5%, whereas that of late apoptotic/necrotic cells increased to 26.2% (Figure 6C). The highest proportion of apoptotic cells was observed at 18 h, and the proportion of late apoptotic/necrotic cells increased to 35.4% (Figure 6D). Therefore, the high level of double positive cells after the 18-h treatment might be a consequence of apoptosis followed by necrosis in the absence of phagocytic cells in this in vitro, experimental model system. The percentage of cells labelled only with PI was also increased in the cultures incubated with TNF-α for 18 h (4.9%) as compared with the untreated control (0.19%) (Figure 6D and 6A).

Together, these data demonstrate that TNF-α induces not only apoptosis, but also necrosis in cultured cardiac myocytes.
DISCUSSION

Different plasma levels of TNF-α, sFas, IL-6 and sIL-6R in DCM and HCM patients

The cardiocytotoxicity of TNF-α observed in a variety of clinical conditions is not only due to the negative inotropic effects of TNF-α, but may also be compounded by TNF-α-induced cell death via apoptosis [11, 24]. The level of plasma TNF-α, an inducer of apoptosis, was significantly higher in the DCM patients. In these patients, an elevated plasma concentration of sFas was also observed. Fas is an apoptosis-signalling surface receptor belonging in the TNF receptor family, which is known to trigger programmed cell death [25]. FasL, the physiological agonist of Fas, is also a transmembrane protein, with homology to the TNF family in its extracellular domain. Fas and FasL have been observed as soluble molecules, in addition, to their membrane-associated forms, suggesting additional complexity as concerns regulation of the apoptotic mechanism. The level of the sFas, similarly an apoptosis-signalling receptor belonging in the TNF receptor family, which is known to trigger programmed cell death [25]. FasL, the physiological agonist of Fas, is also a transmembrane protein, with homology to the TNF family in its extracellular domain. Fas and FasL have been observed as soluble molecules, in addition, to their membrane-associated forms, suggesting additional complexity as concerns regulation of the apoptotic mechanism.

In healthy subjects, but also with those in 19 HCM patients, and found them to be significantly higher in the DCM patients than in either the healthy blood donors or the HCM patients.

In contrast, no elevation of plasma TNF-α or sFas was observed in the HCM patients, whereas significantly higher IL-6 and sIL-6R concentrations were measured than in either the controls or the DCM patients (Figures 3 and 4). IL-6 and sIL-6R together may contribute to cardiac hypertrophy [17, 18, 27]. A complex of IL-6 and IL-6R (either in a membrane-anchored form or in an extracellular soluble form) associates with gp130 to induce its homodimerization. The gp130-mediated signals have a physiological role in cardiomyocyte regulation and, when overstimulated, lead to cardiac hypertrophy [17-19, 27]. A complex of IL-6 and IL-6R (either in a membrane-anchored form or in an extracellular soluble form) associates with gp130 to induce its homodimerization. The gp130-mediated signals have a physiological role in cardiomyocyte regulation and, when overstimulated, lead to cardiac hypertrophy [17-19, 27]. The IL-6/sIL-6R complex can mimic the actions of other members of this cytokine family, including leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1. Cardiotrophin-1 induces myocyte hypertrophy [28]. A hypertrophic response of cultured heart muscle cells to the IL-6/sIL-6R complex has been observed [18]. As the highest level of sIL-6R was observed in HCM patients, it is tempting to speculate that high sIL-6R and IL-6R levels may contribute to cardiac hypertrophy.

When rat cardiomyocytes were analysed with Annexin V after an 18-h incubation with rMuTNF-α, substantial apoptosis was evident. These results lend further support to the
observations that a high concentration of TNF-α, either at the periphery or locally, may cause the apoptosis or even the necrosis observed in heart failure.

**Different expressions of TNF-α, IL-6, Bcl-2 and Bax in tissue samples from DCM and HCM patients**

Apoptosis is critically dependent upon the expression of the Bcl-2 family member proteins, including Bcl-2 itself, which protects cells from apoptotic triggers, and Bax, which promotes apoptosis [29]. It is noteworthy that only a low basal Bcl-2 expression was demonstrated in cardiac tissues from our DCM patients, while the expression of Bax was up-regulated. Previous studies have demonstrated that the biological consequence (i.e. induction or inhibition of apoptosis) of the joint effect of the pro-apoptotic and anti-apoptotic Bcl-2 family members depends on their stoichiometric ratio [30]. The increased ratio Bax/Bcl-2 observed in the myocardial tissues of our DCM patients may reflect the higher susceptibility of the cells to apoptotic stimuli and we therefore presume that the cardiac muscle in these patients is not well protected from apoptotic triggers. At the same time, a considerable local TNF-α expression was detected in these cardiac biopsy specimens. Since TNF-α may be produced by cardiac cells themselves [31], it is possible that the local concentration of TNF-α is higher in the cardiac tissue, and can trigger apoptosis. These findings suggest that the elevated TNF-α levels seen in DCM patients, together with local TNF-α production, may contribute to TNF-α-induced cardiac cell death. IL-6 produced locally might be a consequence of the activation of the cytokine cascade, and the effect of IL-6 on muscle wasting might further influence the patho-mechanism in DCM [32]. In the cardiac tissue samples from the HCM patients, a strong Bcl-2 expression was observed without the presence of TNF-α, IL-6 and Bax (Figure 5). These data indicate an imbalance between pro-apoptotic and anti-apoptotic signals.

In conclusion, these results suggest that the relatively high levels of TNF-α and sFas in DCM patients, together with the local TNF-α and Bax expressions, are signs of apoptotic processes. In contrast, in HCM patients, the high IL-6 level, together with high sIL-6R concentrations, can influence the hypertrophic processes through gp130 signalling. Moreover, there is a sharp difference between the local Bcl-2 expressions, and a considerable upregulation of the anti-apoptotic Bcl-2 is proposed in HCM.

It is obvious that cytokines with apoptotic or hypertrophic effects can not be the sole causative agents of cardiac diseases, but they can influence the magnitude and extent of irreversible tissue damage. The basic pathological condition and the histological impairments in the cardiac tissue, together with the circulatory problems, may determine the cytokine pattern and vice versa. An understanding of the injury process involving cytokines may result in a better mode of intervention and thus in a reduction in cardiac damage.

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