Dilated Cardiomyopathy

Relationship Between Tumor Necrosis Factor-Alpha Production and Oxidative Stress in the Failing Hearts of Patients With Dilated Cardiomyopathy

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OBJECTIVES
This study evaluated oxidative stress in the failing ventricle in patients with dilated cardiomyopathy (DCM).

BACKGROUND
Oxidative stress appears to increase in the failing myocardium and may contribute to ventricular dysfunction in patients with DCM. Tumor necrosis factor-alpha (TNF-alpha), which is expressed in the failing heart, may stimulate oxidative stress.

METHODS
We measured plasma oxidized low density lipoprotein (oxLDL) by sandwich enzyme-linked immunosorbent assay using specific antibodies against oxLDL in the aortic root (AO) and the coronary sinus (CS) in control subjects (n=8) and in 22 patients with DCM and mild congestive heart failure. We also measured the plasma levels of TNF-alpha and angiotensin II.

RESULTS
There was no difference in oxLDL between the AO and CS in control subjects. In contrast, plasma oxLDL was significantly higher in the CS than the AO in patients with DCM, suggesting that the transcardiac gradient of oxLDL reflects oxidative stress in the failing heart in these patients. Plasma TNF-alpha levels were significantly higher in the CS than the AO with a significant positive correlation of the transcardiac gradient of TNF-alpha and the transcardiac gradient of oxLDL. Moreover, a significant negative correlation existed between the transcardiac gradient of oxLDL and left ventricular ejection fraction. The transcardiac gradient of plasma oxLDL was significantly lower in 6 patients who received carvedilol than in 16 patients who did not receive carvedilol.

CONCLUSIONS
These findings indicate that the transcardiac gradient of oxLDL may be a marker of oxidative stress in the heart and that left ventricular dysfunction may be partly due to the oxidative stress in patients with DCM. In addition, TNF-alpha may stimulate oxidative stress in the failing heart in patients with DCM. (J Am Coll Cardiol 2001;37:2086–92) © 2001 by the American College of Cardiology

Recent studies suggest that free radicals are increased in the failing myocardium, and they may be important contributors to the deterioration of decompensating myocardium (1–5). A chronic increase in myocardial oxidative stress is capable of causing subcellular abnormalities, and this may lead to cardiomyopathic changes and depressed contractile function and failure (6–8). Thus, oxidative stress may be an important susceptibility factor for dilated cardiomyopathy (DCM) (9,10). Recently, plasma biochemical markers of oxidative stress have been reported to be increased in patients with congestive heart failure (CHF) including DCM patients (5,11–13). However, there are no reports showing the tissue origins of oxidative stress markers; both poorly perfused peripheral muscles and the failing myocardium may contribute to the increases.

Because free radicals cannot be measured directly in humans, indirect markers of their activity have been used. The most widely used index is the measurement of malondialdehyde by the thiobarbituric acid-reacting substances (TBARS) assay. However, there is a lack of specificity with this assay (14). Recently, a sensitive and specific method of measuring very low concentrations of oxidized low density lipoprotein (oxLDL) was established (15,16). Therefore, in the present study we tested the hypothesis that an elevated transcardiac gradient of oxLDL resulting from oxidative stress in the ventricle contributes to the pathophysiology of DCM. A potentially important stimulus for increased oxidative stress in the failing myocardium is exposure to inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-alpha), which is expressed in the failing myocardium (17–19) and has the ability to stimulate free radical production (20–22). Another important stimulus that increases free radicals is angiotensin II (Ang II) (23–26), which is increased in plasma as well as in the hearts of patients with DCM and CHF.

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Manuscript received November 3 2000; revised manuscript received February 26, 2001, accepted March 14, 2001.
METHODS

Patients. The study population consisted of 22 consecutive patients with mild CHF (left ventricular ejection fraction [LVEF] <45%) and DCM who were admitted to our institution. The diagnosis of DCM was based on patient history, physical examination, electrocardiogram, chest radiography, echocardiography, left ventriculography and coronary angiography. All patients were free of hypertension, ischemic heart disease, valvular heart disease, congenital malformations of the heart or vessels and intrinsic pulmonary, renal or metabolic diseases. Endomyocardial biopsies were obtained to rule out secondary cardiomyopathies caused by viral or other infectious myocarditis, sarcoidosis, amyloidosis or other metabolic heart disease. Patients with secondary DCM were excluded from the study. Among the 22 patients with DCM, there were no patients with diabetes mellitus or hyperlipidemia. We also selected eight age-matched control subjects (aged 34 to 71 years, mean, 54 years) with normal coronary arteries and no coronary spasm by coronary angiography. Among the eight control subjects, there were no patients with diabetes mellitus, hyperlipidemia or hypertension.

Study protocol. All patients were premedicated with an oral dose of diazepam (5 mg), and they rested in bed in the supine position for at least 20 min. Right-sided cardiac catheterization was performed using a 7F Swan-Ganz catheter. Blood samples for measuring plasma levels of TBARS and oxLDL were collected simultaneously from the aortic root (AO), coronary sinus (CS) and femoral vein. Blood samples for measuring plasma levels of Ang II and TNF-alpha were also collected from the AO and CS. A 6F catheter (Goodman Co., Ltd, Nagoya, Japan) for blood sampling was positioned in the CS, and the position of the catheter was confirmed by injection of contrast medium just after blood sampling. Left ventriculography was performed using contrast medium after obtaining hemodynamic measurements and blood samples.

Measurements of neurohumoral factors, thiobarbituric acid reactive substances and oxLDL. Blood for measuring plasma levels of TNF-alpha was transferred to a chilled tube containing ethylenediaminetetraacetic acid (1 mg/ml) and aprotinin (500 kallikrein inactivator U/ml), and then centrifuged at 3,000 rpm for 15 min at 4°C. The plasma obtained was stored at −30°C until assayed. The plasma TNF-alpha level was determined using a commercially available immunoassay (Quantikine HS, R&D Systems, Minneapolis, Minnesota) as previously reported (28).

Plasma Ang II levels were measured using a radioimmunoassay with a specific antibody directed against synthetic Ang II, as previously reported (29).

Blood for measuring plasma levels of the TBARS was transferred to a plain tube, then centrifuged at 3000 rpm for 15 min at 4°C. The plasma obtained was stored at −30°C until assayed. Lipid peroxide concentration was determined by quantifying the TBARS as described previously (30).

Blood for measurement of the plasma levels of oxLDL was transferred to a plain tube and centrifuged at 3,000 rpm for 15 min at 4°C; the plasma obtained was stored at 4°C until it was assayed. Plasma oxLDL levels were measured using a specific immunometric assay for human oxLDL using a kit (Kyowa Medex, Co., Ltd, Tokyo, Japan) (16), which employed a modification of a method previously reported (15). Briefly, this assay system uses two antibodies against human oxLDL, one recognizing a monoclonal antibody against oxidized phosphatidylcholine (DLH3) (15) and the other a polyclonal antibody against human apoprotein B, respectively, and measures oxLDL by sandwiching it between the two antibodies.

Before the assay, the plasma sample was diluted 1/250 with a dilution buffer, and the DLH3-coated plates were washed three times with a phosphate-buffered saline and patted dry. A dilution buffer (100 μl) was added to each well of the plates, and 20 μl of the diluted sample or calibrator was added and mixed with the dilution buffer in the plates. Next, plates were incubated for 2 h at 37°C. After plates were washed five times with washing buffer, horseradish peroxidase-labeled antiapoprotein B-100 goat IgG was added to each well, and the plates were incubated for 1 h at
Clinical characteristics and hemodynamic data. There was no difference in age, gender or plasma levels of total cholesterol, LDL cholesterol, high density lipoprotein cholesterol or triglycerides between the control subjects and patients with DCM. The mean LVEF of the patients with DCM was 31%, and LVEF was significantly lower in patients with DCM than in control subjects (Table 1).

Comparison of plasma concentrations of TBARS and oxLDL in the AO and CS. There was no difference in plasma levels in TBARS between the AO and the CS in 8 control subjects (2.8 ± 0.2 vs. 2.5 ± 0.2 nmol/ml) and in 22 patients with DCM (2.2 ± 0.1 vs. 2.1 ± 0.1 nmol/ml). In the 8 control subjects, no difference existed in the oxLDL level between the AO and CS (Fig. 1) (9.4 ± 0.8 vs. 10.1 ± 1.0 U/ml); in the 22 patients with DCM, the plasma oxLDL level was significantly higher in the CS than in the AO (Fig. 1) (28.6 ± 3.4 vs. 16.4 ± 2.0 U/ml, p < 0.01). No difference existed between the oxLDL level in the AO and in the femoral vein in control subjects or in patients with DCM. The plasma levels of oxLDL in the AO, CS and femoral vein were significantly higher in patients with DCM than in control subjects (Fig. 1).

Relationship between the transcardiac gradient of plasma oxLDL and left ventricular function. Figure 2 shows a significant negative correlation between the transcardiac gradient of plasma oxLDL [(CS − AO) oxLDL] and LVEF. A significant negative correlation existed between the transcardiac gradient of plasma oxLDL [(CS − AO) oxLDL] and LVEF in 30 subjects, consisting of both patients with DCM and the control subjects (r = −0.565, p = 0.0011), and in 22 patients with DCM (r = −0.455, p < 0.05). A significant positive correlation existed between the transcardiac gradient of plasma oxLDL [(CS − AO) oxLDL] and left ventricular end-diastolic volume index in the 30 subjects consisting of the patients with DCM plus control subjects (r = 0.405, p = 0.023) (Fig. 3).

Comparison of plasma levels of oxLDL and neurohumoral factors in patients who received carvedilol and patients who did not receive carvedilol. In the present study, six patients received carvedilol (mean doses, 15 mg/day), which has an antioxidant effect. There was no difference in hemodynamic parameters and other treatments between the two groups (Table 2). There was also no significant difference in the plasma level of oxLDL in the AO or the plasma levels of TNF-alpha or Ang II in the AO and CS between the two groups, but the transcardiac gradient of plasma oxLDL was significantly lower in the 6 patients who received carvedilol than in the 16 patients who did not receive carvedilol (Fig. 4). The transcardiac gradient of plasma TNF-alpha was also significantly lower in the 6 patients who received carvedilol than in the 16 patients who did not receive carvedilol.

Figure 1. Plasma oxidized low density lipoprotein (oxLDL) concentrations in the aortic root (AO), coronary sinus (CS) and femoral vein (FV) in 8 control subjects and in 22 patients with dilated cardiomyopathy. Open columns represent control subjects; closed columns represent patients with dilated cardiomyopathy. *p < 0.05; **p < 0.01 between the value of normal subjects and patients with dilated cardiomyopathy; #p < 0.01 vs. the value of AO by analysis of variance with the Scheffe F test.
Relationship between the transcardiac gradient of plasma TNF-alpha and the transcardiac gradient of plasma oxLDL in patients with DCM. There was no difference in the plasma level of Ang II between the AO and the CS, but plasma levels of TNF-alpha were significantly higher in the CS than those in the AO. No correlation was seen between the plasma levels of TNF-alpha in the AO and CS and the transcardiac gradient of oxLDL, but a significant correlation existed between the transcardiac gradient of plasma TNF-alpha and the transcardiac gradient of plasma oxLDL in 22 patients with DCM (Fig. 5). Table 3 shows the results of univariate and multivariate analyses among 11 variables to assess the factors regulating the transcardiac gradient of plasma oxLDL. According to stepwise multivariate analyses, only the transcardiac gradient of plasma TNF-alpha was a significant independent predictor of the transcardiac increase in plasma oxLDL in 22 patients with DCM and mild CHF.

DISCUSSION

Plasma oxLDL as a sensitive marker of oxidative stress in patients with DCM. Recently, increases in plasma biochemical markers of oxidative stress have been reported in patients with CHF, including patients with DCM (5,11–13). However, there is no report showing the tissue origins of oxidative stress markers except for one report in which pericardial fluid samples were analyzed (31). Thus, it re-

Table 2. Characteristics of 22 Patients with CHF Divided Into Two Groups: Carvedilol Treatment or No Carvedilol Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carvedilol (−)</th>
<th>Carvedilol (+)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>56.2 ± 5.3</td>
<td>59.3 ± 5.4</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>10/6</td>
<td>2/4</td>
<td>NS</td>
</tr>
<tr>
<td>NYHA functional class</td>
<td>13</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>81 ± 5</td>
<td>64.2 ± 4.6</td>
<td>0.063</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>82.5 ± 3.3</td>
<td>82.1 ± 7.6</td>
<td>NS</td>
</tr>
<tr>
<td>CI (liter/min/m²)</td>
<td>2.7 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>12.9 ± 1.4</td>
<td>13.8 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>29.8 ± 2.1</td>
<td>33.7 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Ang II in AO (pg/ml)</td>
<td>17 ± 4.1</td>
<td>52 ± 39</td>
<td>NS</td>
</tr>
<tr>
<td>Ang II in CS (pg/ml)</td>
<td>21 ± 6.4</td>
<td>43 ± 28</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-alpha in AO (pg/ml)</td>
<td>3.5 ± 0.5</td>
<td>4.5 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-alpha in CS (pg/ml)</td>
<td>4.6 ± 0.5</td>
<td>4.5 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>oxLDL in AO (U/ml)</td>
<td>14.9 ± 2.3</td>
<td>20.7 ± 4.5</td>
<td>NS</td>
</tr>
<tr>
<td>oxLDL in CS (U/ml)</td>
<td>30.9 ± 4.4</td>
<td>22.3 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>(CS-AO) oxLDL (U/ml)</td>
<td>16.1 ± 3.2</td>
<td>1.7 ± 1.3</td>
<td>0.015</td>
</tr>
</tbody>
</table>

ACE = angiotensin-converting enzyme; Ang II = angiotensin II; AO = aortic root; CI = cardiac index; CS = coronary sinus; HR = heart rate; LVEDP = left ventricular end-diastolic pressure; LVEF = left ventricular ejection fraction; MBP = mean arterial blood pressure; oxLDL = oxidized low density lipoprotein; TNF-alpha = tumor necrosis factor-alpha.
mains possible that these markers arise from poorly perfused peripheral muscles and/or the failing myocardium. Our findings indicated that there was no difference in plasma oxLDL between the AO and the femoral vein, but the plasma oxLDL level was significantly higher in the CS than in the AO, suggesting that the source of the increase in plasma oxLDL, a marker of oxidative stress, is of myocardial origin rather than peripheral origin in patients with DCM and mild CHF.

Because the evaluation of oxidative stress in the heart is difficult in vivo, we measured the transcardiac oxLDL gradient as a marker of oxidative stress in the heart. We also measured plasma TBARS, but there was no difference between the AO and the CS. In the present study, there was a significant increase in plasma oxLDL from the AO to the CS in patients with DCM, suggesting: 1) plasma oxLDL is a sensitive maker of oxidative stress, and 2) that there is an increase in the production of free radicals such as superoxide radical and/or a decrease in free radical scavengers in the failing hearts of patients with DCM. Moreover, the increased transcardiac gradient of plasma oxLDL was negatively correlated with LVEF. Our findings are consistent with those of experimental studies in which free radicals depressed myocardial contractile function (6–8).

**TNF-alpha as a potential stimulator of oxidative stress in patients with DCM.** A potentially important stimulus for increased oxidative stress in the myocardium is exposure to inflammatory cytokines, such as TNF-alpha which is expressed in the failing myocardium but not in the healthy heart (17–19) and which has the ability to stimulate free radical production (10–22). The other important stimulus for increased free radicals is Ang II (23–26), which is increased in plasma as well as in the hearts of patients with DCM and CHF, and which has the ability to stimulate free radical production.

In the present study, a transcardiac increase in TNF-alpha was observed in patients with DCM and mild CHF, suggesting that TNF-alpha is produced in myocardial tissue and the source of increased plasma TNF-alpha is partly the failing heart. Although no correlation was seen between the plasma levels of TNF-alpha in the AO and CS and the transcardiac gradient of oxLDL, the transcardiac gradient of TNF-alpha significantly correlated with the transcardiac gradient of oxLDL, indicating that the local production of TNF-alpha in the heart stimulates oxidative stress in the failing heart with DCM. Previous studies reported that myocardial overexpression of TNF-alpha causes ventricular dilation, ventricular dysfunction and interstitial fibrosis like DCM (32,33), suggesting the local expression of TNF-alpha in the heart plays a causal role in the pathogenesis of DCM. Taken together with the present study, oxidative stress induced by the local production of TNF-alpha in the heart may cause left ventricular dysfunction in patients with DCM.

In the present study, there was no difference in the plasma level of Ang II between the AO and the CS there was no relation between plasma levels of Ang II in the AO, CS and the transcardiac gradient of Ang II and the transcardiac gradient of oxLDL. In contrast to TNF-alpha, Ang II is not only produced but also extracted through the heart (34), so it is difficult to evaluate the tissue level of Ang II in the

**Figure 5.** Correlation between the transcardiac gradient of plasma tumor necrosis factor-alpha (TNF-alpha) and the transcardiac gradient of plasma oxidized low density lipoprotein (oxLDL) in patients with dilated cardiomyopathy. AO = aortic root; CS = coronary sinus.

**Table 3.** Univariate and Multivariate Linear Model of Transcardiac Gradient of oxLDL in 22 Patients With DCM

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate Correlation Coefficient</th>
<th>p Value</th>
<th>Multivariate Beta-Coefficient (SE)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NYHA functional class</td>
<td>−0.033</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>−0.039</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>−4.55</td>
<td>0.0332</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang II in AO (pg/ml)</td>
<td>−0.138</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang II in CS (pg/ml)</td>
<td>−0.97</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CS-AO) Ang II (pg/ml)</td>
<td>0.172</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-alpha in AO (pg/ml)</td>
<td>−0.125</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-alpha in CS (pg/ml)</td>
<td>−0.086</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CS-AO) TNF-alpha (pg/ml)</td>
<td>0.544</td>
<td>0.0088</td>
<td>8.4 (2.89)</td>
<td>0.0088</td>
</tr>
<tr>
<td>Treatment (treatment = 1)</td>
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<tr>
<td>ACE inhibitors</td>
<td>−0.123</td>
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<tr>
<td>Carvedilol</td>
<td>−0.509</td>
<td>0.0156</td>
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</tbody>
</table>

ACE = angiotensin-converting enzyme; Ang II = angiotensin II; AO = aortic root; CS = coronary sinus; LVEF = left ventricular ejection fraction; NYHA = New York Heart Association; TNF-alpha = tumor necrosis factor-alpha.
failing heart. Therefore, we cannot deny the potential role of local ANG II as a stimulator of oxidative stress in the failing heart with DCM.

**Clinical Implications.** Several studies have provided strong evidence that enhanced lipid peroxidation and oxygen free radical damage persists in patients with CHF (1–8). Although the precise cellular sources of oxygen free radicals and the mechanisms involved in initiating the oxidative stress that occurs in patients with CHF remain unknown, our findings suggest that failing myocardium is one of the sources of the increase in free radicals in patients with DCM. It is conceivable that oxygen free radicals produced by the myocardium contribute to myocardial cellular toxicity and damage and to apoptosis (1,2). It has been proposed that the link among oxygen free radical formation, cardiac remodeling and apoptosis involves the activation of specific transcription factors in cardiac myocytes. In this respect, antioxidants have been proposed to attenuate the oxygen free radical–derived transcriptional events that are responsible for cardiac remodeling and apoptosis. In the present study, there was a significant positive correlation between the transcardiac gradient of plasma oxLDL and left ventricular end-diastolic volume index. Six patients received carvedilol, which has an antioxidant effect, and the transcardiac gradients of plasma oxLDL and TNF-alpha were significantly lower in patients receiving carvedilol therapy than in patients not receiving carvedilol, suggesting that carvedilol may reduce the oxidative stress of the failing heart of DCM patients. However, we cannot deny the possibility that carvedilol reduced the transcardiac gradient of oxLDL through its beta-blocking, metabolic and remodeling effects as opposed to its antioxidant properties. Therefore, the effects of antioxidant drugs, including other beta-blockers such as metoprolol on the serial changes in the transcardiac gradient of oxLDL need to be studied.

**Study Limitations.** In the present study, we evaluated patients with DCM and mild CHF. In patients with severe CHF, ANG II and TNF-alpha, which are more activated in the failing heart than in mild CHF patients, may stimulate oxidative stress and exacerbate the transcardiac gradient of oxLDL. Further studies are needed to evaluate the relationship between the severity and/or etiology of CHF and the transcardiac gradient of oxLDL.

**Conclusions.** In conclusion, this is the first study, to our knowledge, to demonstrate that the transcardiac gradient of oxLDL may be a marker of oxidative stress in the heart and that left ventricular dysfunction may be partly due to oxidative stress in patients with DCM. Moreover, the present study indicated that TNF-alpha may stimulate oxidative stress in the failing heart in patients with DCM.

**Acknowledgments**

We wish to thank Ms. Ikuko Sakaguchi for her excellent technical assistance. We also express thanks to Mr. Daniel Mrozek for assistance in preparing the manuscript.

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**References**