Expression of Cell Adhesion Molecules in Dilated Cardiomyopathy

Evidence for Endothelial Activation in Inflammatory Cardiomyopathy

Michel Noutsias, MD; Bettina Seeberg, MD; Heinz-Peter Schultheiss, MD; Uwe Kühl, PhD

Background—Dilated cardiomyopathy (DCM) is pathogenically linked to inflammatory cardiomyopathy (InfCM), which is characterized by intramyocardial infiltration. The transendothelial migration of immunocompetent cells is mediated by cell adhesion molecules (CAMs).

Methods and Results—We investigated the expression pattern of CAMs (immunoglobulin superfamily, 32 selectins, and β1- and β2-integrins) in endomyocardial biopsies from DCM patients (n = 152; left ventricular ejection fraction < 40%) using immunohistochemistry. Whereas few specimens obtained at autopsy (controls; n = 14) presented enhanced expression regarding single endothelial CAMs (human leukocyte antigen [HLA] class I, 7%; HLA-DR, 14%; CD29, 14%), none demonstrated concurrent abundance of > 3 CAMs (inflammatory endothelial activation), nor did any control tissue prove positive for InfCM (> 7.0 CD3+ lymphocytes per 1 mm²). In comparison, 64% (n = 97) of the DCM biopsies were evaluated positive for InfCM and 67% (n = 101) for inflammatory endothelial activation, respectively. Whereas expression of HLA class I, HLA-DR, intercellular cell adhesion molecule-1, and CD29 was distributed homogeneously within a patient’s serial sections, immunoreactivity of vascular cell adhesion molecule-1, lymphocyte function antigen-3, and the selectins was accentuated on single vascular endothelia. Sixty-six percent of the DCM biopsies presented CD29 abundance also within the extracellular matrix and the sarcolemma. CD62P and CD62E were present in 16% and 40% of the DCM patients, respectively. Endothelial CAM representatives correlated with one another (P < 0.05), except for CD62P with HLA. Endothelial CAM expression correlated with intramyocardial infiltrates phenotyped by the corresponding counterreceptors.

Conclusions—Inflammatory endothelial activation is present in 67% of DCM patients. Because CAM expression correlates with the immunohistological diagnosis of InfCM and counterreceptor-bearing intramyocardial infiltrates, evaluation of endothelial CAMs might be of diagnostic significance in InfCM. (Circulation. 1999;99:2124-2131.)

Key Words: cardiomyopathy n diagnosis n cell adhesion molecules n endothelium n immune system n immunohistochemistry

Inflammatory cardiomyopathy (InfCM) constitutes a specific entity of cardiomyopathy, being characterized by idiopathic heart failure with evidence for intramyocardial inflammation.1 The diagnosis of inflammatory heart disease has been a troublesome topic since its introduction, and the applied diagnostic criteria vary even among leading centers.2 The Dallas criteria3 have proved most suitable for the acute stage of myocarditis.4 With the more sensitive and specific immunohistological approach, InfCM was revealed in about the moiety of dilated cardiomyopathy (DCM) patients.5,6 Although these immunohistological criteria were cited in the WHO/ISFC report,1 the definitive diagnostic criteria remain to be specified, possibly by future task forces.2 A “nonhistological marker,” being independent of focally clustered infiltrates as to the distribution pattern, is not prone to sampling error7 but concurrently being associated with intramyocardial infiltrates, would be favorable.8

Until now, experimental investigations and diagnostic procedures in inflammatory heart disease have focused on intramyocardial infiltrates. However, endothelial cells are probably infected before cardiotropic viruses invade the myocardium.9,10 The phenotypic pattern of endothelial cells is reportedly altered in DCM hearts with respect to cell adhesion molecules (CAMs).9,11–13

The network of endothelial adhesion receptors and their specific ligands on circulating immune cells orchestrates the sequence of initial rolling mediated by selectins, the subsequent firm adhesion, and ultimately the transendothelial migration of immunocompetent cells directed by integrins and members of the immunoglobulin superfamily, thus conferring spatial, temporal, and leukocyte-type selectivity to the
recruitment process. In general, CAM expression is regulated by proinflammatory cytokines in terms of either enhancement of baseline expression or de novo induction. Whereas vascular cell adhesion molecule (VCAM)-1 and the selectins CD62E and CD62P are expressed exclusively on activated endothelial cells, the tissue distribution of lymphocyte function antigen (LFA)-3, intercellular cell adhesion molecule (ICAM)-1, and human leukocyte antigen (HLA) molecules comprises additionally interstitial cells (eg, immunocompetent infiltrates, histiocytes, dendritic cells, and fibroblasts). Furthermore, β2-integrins (CD29 and very late activation antigen [VLA]-4) are also expressed within components of the extracellular matrix and on the sarcolemma of regenerating skeletal myocytes. β1-Integrins are expressed by immunocompetent cells (CD18, pan-leukocyte marker; LFA-1, activated lymphocytes; Mac-1, monocytes and macrophages).

The objectives of the present study were to investigate the expression pattern of CAMs (immunoglobulin superfamily, selectins, and β1- and β2-integrins) in endomyocardial biopsies from DCM patients and to conclude the diagnostic and pathogenic significance of CAMs in InfCM.

Methods

Study Group
We enrolled 152 patients (men, n = 104; women, n = 48; age, 47.2 ± 17.4 years) clinically presenting with DCM (left ventricular ejection fraction [LVEF] < 40%). Duration of symptoms was > 6 months (range, 6 months to 10 years) without any discernible clinical history of viral infection preceding or related to the onset of symptoms of heart failure. All patients underwent ECG, echocardiography, and catheterization of the left side of the heart (ventriculography, selective coronary angiography, and assessment of both left and right ventricular hemodynamic functions). Secondary etiopathogenesis (systemic hypertension, ischemic, and valvular and congenital heart diseases) was excluded.

Specimens obtained from the right ventricular septum at autopsy (n = 14; men, n = 8; women, n = 6; age, 47.5 ± 17.5 years) within 48 hours after noncardiac death (trauma, n = 2; malignancy, n = 6; intoxication, n = 4; rupture of abdominal aortic aneurysm, n = 1; pneumonia, n = 1) served as controls. To investigate the relevance of our controls with respect to a possible postmortem degradation of CAMs, we subjected 7 tissues obtained from the right ventricular septum of DCM explants (men, n = 5; women, n = 2; age, 42.4 ± 16.3 years; LVEF < 25%) to autolysis at room temperature and compared CAMs immunoreactivity at days 0 (immediate freezing after explantation), 1, 2, and 3 (after autolysis). Informed consent was obtained from all patients.

No significant differences in age or sex were calculated between the autopsied control group, transplanted DCM patients, and DCM patients from whom the biopsies were obtained (P < 0.05).

Sample Preparation
Multiple endomyocardial biopsies (≥6 per patient) from the right ventricular septum were obtained by standard percutaneous transvenous right femoral approach with a Cordis biopette. For conventional histological evaluation following the Dallas criteria, 2 biopsies were fixed in 10% formalin. For immunohistological evaluation, the remaining specimens were embedded in Tissue Tec (SLEE) and immediately snap-frozen in methylbutane cooled in liquid nitrogen at −70°C. Biopsy specimens were cut serially into cryosections of 5 μm thickness, which were placed on 10% poly-L-lysine–precoated slides. Depending on availability, 6 to 9 sections from a single biopsy were analyzed for each antibody per patient. The coded slides were examined in a blinded fashion.

Light Microscopy
Specimens were stained with hematoxylin and eosin according to standard protocols. The diagnostic procedure for active and borderline myocarditis was based on the Dallas criteria: Borderline myocarditis was confirmed by the presence of increased mononuclear infiltrates in the absence of myocytolysis, whereas active myocarditis also required unequivocal evidence for myocytolysis adjacent to mononuclear infiltrates.

Immunohistochemical Staining Procedure
After fixation in cold acetone for 10 minutes and subsequent air drying, endogenous peroxidase activity was quenched by incubating cryosections with 0.3% H2O2 in PBS for 20 minutes. After 3 rinses in PBS, cryosections were incubated with the appropriately diluted monoclonal mouse antibody in PBS containing 5% heat-inactivated FCS (saturation of unspecific protein binding sites) for 1 hour in a humidified chamber. The slides were then rinsed 3 times in PBS and then incubated with the peroxidase-conjugated polyclonal rabbit–anti-mouse antibody (dilution 1:200; Dianova) for 45 minutes in a humidified chamber. After 3 rinses in PBS, immunoreactive staining was developed by use of 3-amino-9-ethylcarbazole (Merck) as chromogenic substance being converted by peroxidase to a red precipitate. The slides were finally mounted with Kaiser’s gelatin (Merck). The antibodies were purchased from Dianova, except for anti–ICAM-1 and anti-CD62E (Serva).

Immunohistochemical Quantitative Evaluation of Infiltrates
Immunohistochemically stained cells were counted per high-power field (400-fold magnification, which was equivalent to 0.28 mm2) by use of the Leica MDRD microscope in all available fields (>10 fields per antibody), and the mean cell counts per high-power field were computed. Figure 1 demonstrates immunostained infiltrates.

Semi-quantitative Evaluation of Inflammatory Activation of Endothelial Cells
Immunoperoxidase staining of endothelial cells was graded as follows: grade 0, no discernable immunoreactivity; grade 1, faint CAM staining; grade 2, enhanced CAM expression; and grade 3, strongly abundant CAM immunoreactivity.

CAMs known to be orthogradely almost absent and de novo induced during inflammation (LFA-3, VCAM-1, CD62E, and CD62P) were scored positive when immunoperoxidase staining exceeded grade 0. In contrast, constitutively expressed CAMs (HLA class I, HLA-DR, ICAM-1, and CD29) were scored positive if immunoreactivity exceeded grade 1.

In accordance with results published elsewhere and our overall experience of >600 cases, biopsies demonstrating concurrent endothelial activation with respect to ≥3 CAMs (HLA class I, HLA-DR, ICAM-1, VCAM-1, LFA-3, CD62E, or CD62P) were considered positive for inflammatory endothelial activation. Representative endothelial CAM immunostainings are presented in Figure 2.

Statistical Analysis
Statistical analysis was performed with JMP Statistical Discovery Software, version 3.2.2 (SAS Institute, Inc). Because normal distribution was excluded regarding all parameters conducting the Shapiro-Wilk test (P < 0.05), exclusively nonparametric tests were performed. Quantitative data were correlated by use of the Spearman ρ analysis; quantitative data were compared with qualitative data by use of the Wilcoxon/Kruskal-Wallis test on rank sums; and qualitative data were compared by use of the χ2 test (Pearson’s correlation coefficient). The honestly significant difference for multiple comparisons of all pairs was calculated according to the Tukey–Kramer analysis. CAM immunoreactivity in explanted tissues subjected to autolysis was analyzed through MANOVA for repeated measures (time as term of effect). A value of P < 0.05 was considered statistically significant.
Results

Of the specimens obtained at autopsy, none presented by conventional histological or by immunohistological evaluation any evidence for increased mononuclear infiltrates or inflammatory endothelial activation. According to the Dallas criteria, 3 of the DCM cases (2%) were diagnosed as borderline myocarditis (increased mononuclear infiltrates without myocytolysis); no biopsy specimen demonstrated myocytolysis. In comparison, a substantially higher frequency of lymphocytic infiltration was diagnosed by means of immunohistology: 64% (n=97) of the DCM specimens met the criterion of InfCM (>7.0 CD3+ lymphocytes per 1 mm²). Specimens from DCM patients and the control group differed significantly with respect to CAM expression, except for CD62P (Table 1). Inflammatory endothelial activation (ie, concurrent abundant expression of ≥3 CAMs) was evaluated in 67% (101) of the DCM biopsies (Table 2). The 2 diagnostic approaches (increased intramyocardial CD3+ infiltrates and inflammatory endothelial activation) correlated significantly with one another (P=0.0004). A noteworthy finding was that all 3 biopsies diagnosed as borderline myocarditis according to the Dallas criteria were also positive with respect to the criteria of increased lymphocytic and inflammatory endothelial activation. LVEF did not correlate with the immunohistological diagnosis of InfCM (P=0.25) or with the criterion of inflammatory endothelial activation (P=0.81).

Intramyocardial infiltrates demonstrated both focal and diffuse patterns of distribution (Figure 1). In 13 cases (9%), foci of infiltrates closely adjacent to cardiomyocytes, suggestive of myocytolysis, were noted (Figure 1b). Interestingly, the 3 cases diagnosed as borderline myocarditis according to the Dallas criteria were among these. Although perivascular clusters of infiltrates were observed occasionally (Figure 1), no obvious spatial relationship between intramyocardial infiltrates and endothelial CAM expression was observed.

The grade of immunoreactivity regarding HLA class I, HLA-DR, ICAM-1, and CD29 was equally distributed within all sections obtained from a single patient, regardless of focally clustered immunocompetent infiltrates (Figure 2a). The expression pattern of these homogeneously distributed CAMs comprised both larger vessels and capillaries (Figure 2a and 2c through 2f). In contrast, VCAM-1, VLA-4, LFA-3,
Correlations of virtually all studied CAM representatives in processes. This notion is supported by the significant accuracy of the criteria proposed here. Our data substantiate the hypothesis of a chronic active inflammatory process being intimately linked to the pathogenesis of DCM. Notably, investigations by Marijaniowksi et al and Devaux et al attributed this observation to the hypothesized absence of acute inflammatory responses in failing myocardium. In light of our data and results by Ino et al, this discrepancy of observations is due instead to multiple bivariate analysis (Figure 3). Moreover, enhanced expression of single CAM representatives may also occur in diseases without primary cardiac involvement, as our data on autopsies from noncardiac death causes (Table 1) and further investigations might imply. In fact, interindividual variances of CAM expression have been reported even in nonfailing hearts. Therefore, the proposed score here requiring the concurrent abundance of at least 3 different endothelial CAMs confers certainty about an intramyocardial inflammatory process, because this criterion was not met in any of the controls. The reliability of our controls obtained even without respect to CAMs demonstrating low expression (CD6E2, CD6P).

When stimulated with cytokines, endothelial cells express CAMs dynamically and finally shed the adhesion receptors in vitro. The peaks of expression appear for CD6E2 after 2 to 4 hours, for VCAM-1 after 4 to 8 hours, and for ICAM-1 after 6 to 72 hours after stimulation. Whereas de novo expressed CAMs (selectins and VCAM-1) persist at higher levels after the peak, constitutively expressed adhesion receptors (ICAM-1) decline after having passed their peaks, constitutively expressed adhesion receptors (ICAM-1) persist at higher levels after the peak. Provided that these experimental insights may be extrapolated to in vitro conditions, expression of selectins, which was the case in vivo conditions, expression of selectins, which was the case in 16% (CD6P) and 40% (CD6E2) of our DCM patients, in 16% (CD6P) and 40% (CD6E2) of our DCM patients, may indicate an early stage of cytokine release and thus may account for determination of inflammatory stage in InfCM. Notably, investigations by Marijaniowksi et al and Devaux et al failed to demonstrate immunoreactivity of VCAM-1 and CD6E2 in explants of both DCM and myocarditis patients. Devaux et al attributed this observation to the hypothesized absence of acute inflammatory responses in failing myocardium. In light of our data and results by Ino et al, this discrepancy of observations is due instead to

| TABLE 2. Inflammatory Endothelial Activation in DCM |  |
| --- | --- | --- | --- | --- | --- |
| Antigens | No Endothelial Activation | Endothelial Activation |
| | (n=51) | n | % | n | % | P |
| HLA class | | 17 | 33 | 60 | 60 | 0.0072 |
| I-DR | | 20 | 39 | 74 | 73 | <0.0001 |
| ICAM-1/CD54 | | 2 | 4 | 82 | 81 | <0.0001 |
| VCAM-1/CD106 | | 9 | 18 | 93 | 92 | <0.0001 |
| LFA-3/CD58 | | 6 | 12 | 85 | 84 | <0.0001 |
| CD6E2 | | 6 | 12 | 55 | 55 | <0.0001 |
| CD6P | | 2 | 4 | 22 | 22 | 0.0070 |
| CD29 | | 7 | 14 | 93 | 92 | <0.0001 |
| VLA-4/Cdw49 | | 3 | 6 | 81 | 80 | <0.0001 |

Biopsies from DCM patients (n=152) evaluated positive for inflammatory endothelial activation (n=101); concurrent abundance of at least 3 CAMs demonstrated significantly higher frequencies of abundance with respect to all studied CAMs compared with biopsies not meeting the criteria of endothelial activation. The table depicts absolute values (n) and frequencies (%) of endothelial CAMs abundance.
methodical pitfalls, especially since we also found expression of both VCAM-1 (57%) and CD62E (43%) in terminally failing DCM explants (LVEF, 25%).

CD29, the common β1-integrin chain, is reportedly expressed by endothelia, by immunocompetent cells, within components of the extracellular matrix, 14,17,18 and by regenerating skeletal myocytes. 19 Interestingly, whenever CD29 immunoreactivity was enhanced on endothelia and within the extracellular matrix, CD29 expression was also noted on the sarcolemma of cardiomyocytes, which was the case in 66% of our DCM study group. To the best of our knowledge, this is the first investigation reporting CD29 expression on the sarcolemma of adult cardiomyocytes. Notably, except for CD29, CAM immunoreactivity was never observed on the sarcolemma in DCM/InfCM hearts. This is consistent with other investigators, who reported ICAM-1 expression on the sarcolemma in acute myocarditis but not in DCM/InfCM. 12,21,22 Enhanced expression of CD29 within components of the extracellular matrix in InfCM may contribute to an adhesive environment into which inflammatory cells are more readily recruited and retained;16 may furthermore participate in a more profound anchorage of cardiomyocytes serving as laminin, fibronectin, and vitronectin receptor;19 and may finally, like the suggested significance of the sarcolemmal ICAM-1 expression in acute myocarditis,21,22 contribute to an adhesion-mediated cytotoxic attack of immune effector cells to CD29-expressing cardiomyocytes.

Endothelial CAMs and Intramyocardial Infiltrates

With respect to the specific ligand-counterreceptor interactions, ICAM-1 interacts with both LFA-1 and Mac-1, VCAM-1 with VLA-4, LFA-3 with CD2, HLA class I with CD8, and HLA class II with CD4. 14 Our data confirm the hypothesis that expression of endothelial CAMs constitutes an essential prerequisite for the transendothelial migration of circulating immunocompetent cells, because inflammatory endothelial activation correlated with the diagnosis of InfCM based on CD3+ lymphocytes. This notion is best illustrated by the significant correlations of CAMs expression and counterreceptor-bearing infiltrates (Figure 4). However, the most intriguing aspect of a functional dependence of intramyocardial infiltration on CAM expression, namely a spatial relationship between perivascular foci (infrequently observed in InfCM) and accentuated CAMs abundance on adjacent endothelia, was not observed. Nonetheless, such phenomena were not necessarily expected, because the follow-up of a dynamic process cannot be guaranteed by investigating single biopsies obtained at a certain point in time. On the other hand, the homogeneous distribution pattern of ICAM-1, HLA, and CD29 within all serial sections from a certain patient may have accounted for the failure to observe such phenomena. However, such a relationship cannot be excluded by the present study, because multiple immunostaining techniques are needed to explore this issue.

Interestingly, when CAM expression in explanted DCM tissues subjected to autolysis is compared, the nonhomogeneously distributed CAMs (VCAM-1, LFA-3, CD62E, CD62P, and VLA-4) presented higher variances (not in a time-dependent manner but stochastically) than the homogeneously distributed CAMs (ICAM-1, HLA, and CD29), the latter being expressed virtually steadily in all biopsies from a single subject. We therefore conclude that this observation is probably due to sampling effects, which may predominantly affect the nonhomogeneously distributed CAMs, not being broadly expressed on capillaries but rather on venules, which are infrequently present in biopsies. 21 This issue appears exceptionally important in light of the considerable sampling error associated with the diagnosis of InfCM based on intramyocardial infiltrates. 7 Therefore, the requirements for a reliable “nonhistological marker” 8 may be very well met by the homogeneously distributed endothelial CAMs. Moreover, considering that induction of HLA precedes intramyocardial infiltration, 23 the abundance of endothelial CAMs may be interpreted as inflammatory intramyocardial activation even in the absence of significant infiltrates. Finally, the pathogenic significance of merely increased CD3+ lymphocytic infiltrates remains unclear, because this attribute does not necessarily specify activated or cytotoxic cells. 24

In conclusion, CAMs constitute relevant diagnostic targets in InfCM and might be considered for the criteria elaborated on by future WHO/ISFC task forces. 2

Figure 3. Correlation analysis of endothelial CAM immunoreactivity. Endothelial CAM immunoreactivity correlated with one another, except for HLA class I and HLA-DR compared with CD62P. Horizontal bars illustrate Spearman ρ correlation coefficient.
Figure 4. Correlation of endothelial CAM expression with counterreceptor-phenotyped infiltrates. Endothelial CAM immunoreactivity correlated with counterreceptor-bearing infiltrates per high-power field (Wilcoxon/Kruskal-Wallis test with post hoc Tukey-Kramer analysis comparing all pairs; α level, 0.05). Quintile box plots (left rectangles) depict 10th, 25th, 50th, 75th, and 90th percentiles as horizontal lines. Comparison circles (rectangles on right) for significantly different means do not intersect. Means are not significantly different if comparison circles are nested or intersect by angle >90°. Gray comparison circles refer to baseline endothelial expression level of corresponding CAMs (most left quintile boxes). Median and range (25th and 75th percentiles in brackets) of cells per high-power field are depicted above quintile boxes of corresponding grade of CAM immunoreactivity. Overall mean of counted cells per high-power field is indicated by continuous horizontal black line crossing both rectangles.
TABLE 3. Endothelial Adhesion Molecule Expression in Specimens Subjected to Autolysis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA class I</td>
<td>2.2</td>
<td>2.2</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>2.0</td>
<td>2.0</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>ICAM-1/CD54</td>
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<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>VCAM-1/CD106</td>
<td>0.5</td>
<td>0.7</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>LFA-3/CD58</td>
<td>0.7</td>
<td>0.5</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>CD62E</td>
<td>0.5</td>
<td>0.5</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>CD62P</td>
<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CD29</td>
<td>2.3</td>
<td>2.2</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>VLA-4/CDw49d</td>
<td>0.5</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
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</table>

Specimens (n=7) obtained from explanted DCM hearts were subjected to autolysis at room temperature for 3 days. CAM immunoreactivity was evaluated at days 0, 1, 2, and 3 after explantation. The overall means calculated by MANOVA were not significant with “time” used as term of effect (P>0.05).

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