Studies of Endothelial Function

Endothelial Dysfunction of Peripheral Arteries in Patients With Immunohistologically Confirmed Myocardial Inflammation Correlates With Endothelial Expression of Human Leukocyte Antigens and Adhesion Molecules in Myocardial Biopsies

Katja B. Vallbracht, MD, Peter L. Schwimmbeck, MD, Bettina Seeberg, MD, Uwe Kühl, MD, PhD, Heinz-Peter Schultheiss, MD

Berlin, Germany

OBJECTIVES   The aim of this study was to investigate whether myocardial inflammation (MC) and endothelial activation are associated with clinically detectable endothelial dysfunction.

BACKGROUND   In patients with MC, immunohistologic evaluation of myocardial biopsies demonstrates a cellular infiltrate of lymphocytes in the myocardium and endothelial activation, as indicated by enhanced expression of human leukocyte antigen (HLA)-1, HLA-DR and intercellular adhesion molecule (ICAM)-1. This chronic inflammatory process may be associated with endothelial dysfunction.

METHODS   In 65 patients with suspected MC, endothelial function of the radial artery was noninvasively assessed. By means of high-resolution ultrasound, diameter changes in response to reactive hyperemia (endothelium-dependent), as compared with glyceroltrinitrate (endothelium-independent), were analyzed. In the myocardial biopsies, MC was confirmed by immuno-histology in 53 patients; 12 patients with normal myocardial biopsies served as controls. Endothelial expression of HLA-1, HLA-DR and ICAM-1 was semiquantitatively evaluated by immunohistology. To minimize other factors influencing endothelial function, patients with coronary artery disease, diabetes, severely impaired left ventricular function or more than one arteriosclerotic risk factor were excluded from this study.

RESULTS   Endothelial function, as determined by flow-mediated vasodilation (FMD), in patients with MC was impaired (FMD_{MC} 4.28%), as compared with controls (FMD_{Co} 10.10%). The severity of endothelial dysfunction in patients with MC correlated significantly with the extent of endothelial expression of HLA-1, HLA-DR and ICAM-1 in myocardial biopsies. Endothelium-independent vasodilation was not affected by MC or endothelial activation.

CONCLUSIONS   Myocardial inflammation is associated with endothelial dysfunction of peripheral arteries. The severity of endothelial dysfunction correlates with the extent of endothelial activation.   (J Am Coll Cardiol 2002;40:515–20) © 2002 by the American College of Cardiology Foundation

Endothelial function plays a central roll in the development of vascular disease and represents a marker of prognostic relevance; endothelial dysfunction in coronary artery disease is associated with increased development of adverse coronary events (1,2). After heart transplantation, endothelial dysfunction anticipates the development of transplant vasculopathy and transplant failure (3–5). Endothelial dysfunction has been observed in association with acute systemic inflammatory immune responses (6,7). It has been correlated with levels of C-reactive protein (8). Furthermore, clinical studies have shown that endothelial function can be restored by suppression of inflammation in patients with systemic vasculitis (9).

Immunohistologic evaluation of myocardial biopsies in patients with myocardial inflammation (MC) demonstrates a myocardial lymphocyte infiltrate and endothelial activation that can be detected by enhanced expression of cell adhesion molecules (CAMs) (10,11). Thus, the vessel wall is involved in the inflammatory process. Cell adhesion molecule expression is generally regulated by proinflammatory cytokines that can effect not only the local vasculature in the heart, but also, systemically, the peripheral vessels. A correlation between coronary and peripheral endothelial function has been demonstrated for other diseases (12). The aim of the study presented here was to investigate whether the inflammatory process in the myocardium leads to a systemic deterioration of endothelial function. Furthermore, we aimed to elucidate if the severity of endothelial dysfunction in patients with MC correlates with the extent of

Abbreviations and Acronyms

CAM	= cell adhesion molecule
Co	= control group
ECG	= electrocardiogram
EF	= ejection fraction
FMD	= flow-mediated vasodilation
GTN	= glyceroltrinitrate
GTN-MD	= glyceroltrinitrate-mediated vasodilation
HLA	= human leukocyte antigen
ICAM	= intercellular adhesion molecule
MC	= myocardial inflammation

endothelial activation, as detected by enhanced expression of human leukocyte antigen (HLA)-1, HLA-DR and intercellular adhesion molecule (ICAM)-1 in myocardial biopsies.

METHODS

Study population. We included 65 consecutive patients with suspected MC, considering clinical symptoms and noninvasive testing as electrocardiogram (ECG), echocardiography or spiroergometry. When coronary artery disease was excluded by angiography, myocardial biopsies were obtained to establish a diagnosis. According to the findings in the myocardial biopsies, the patients were divided into a group with MC and a control group (Co). In 53 patients, MC was confirmed by immunohistology in myocardial biopsies; 12 patients had normal myocardial biopsies (Co). To minimize other confounding factors on endothelial dysfunction, patients with coronary artery disease (1,2), diabetes (13), more than one risk factor for arteriosclerosis (13–16), overt arteriosclerosis or other severe disease, like malignancy, autoimmune disorders, renal or hepatic failure or recent operations, were excluded from this study. As heart failure is known to affect endothelial function (17–22), we excluded patients with severely impaired left ventricular contractility (ejection fraction [EF] <35%). At the time of the study, the majority of patients was already on cardiovascular medication, which is known to influence endothelial function (23–25). To exclude acute effects, all cardiovascular medication was ceased 12 h to 48 h before the study, depending on half-life. Patients were required to have sinus rhythm. Of 74 patients screened, 9 had exclusion criteria; 65 met the inclusion criteria.

Informed consent was obtained from all patients; the study protocol was approved by the local Ethics Committee of the Free University of Berlin.

Myocardial biopsies. SAMPLE PREPARATION. Endomyocardial biopsies from the right ventricular septum were obtained by standard percutaneous transvenous femoral approach with a standard bioptome. For immunohistologic evaluation, the samples were prepared as published previously (11); biopsies were embedded in Tissue Tec (Sakura Finetek Europe, Zouterwoude, The Netherlands), immediately snap-frozen in methylbutane, cooled in liquid nitrogen

at −70°C and then serially cut into cryosections of 5 μm thickness. Six to nine sections from a single biopsy were analyzed for each antibody per patient. After fixation in cold acetone, endogenous peroxidase activity was quenched by incubating the cryosections with 0.3% H_2O_2 in phosphate buffered solution (Dulbeco, Seromed Biochrom, Berlin, Germany). The cryosections were then incubated with monoclonal mouse antibody in PBS, containing 5% heat-inactivated fetal calf serum (Seromed Biochrom) (saturation of unspecific protein binding sites). The slides were incubated with peroxidase-conjugated polyclonal rabbit antimouse antibody, dilution 1:200 (Dianova, Hamburg, Germany). Immunoreactive staining was developed by use of 3-amino-9-ethylcarbazole (Merck, Germany), being converted by peroxidase to a red precipitate. The antibodies used are commercially available from Dianova (anti-HLA) and Serva (anti-ICAM).

IMMUNOHISTOLOGIC EVALUATION. Immunohistologically stained leucocytes were counted per high-power field (400-fold magnification, 0.28 mm²) by use of a Leica (Bensheim, Germany) MDRD microscope in all available fields (>10 fields per antibody). The mean cell counts per high-power field were computed. Myocardial inflammation was confirmed in myocardial biopsies, if more than seven CD3+ lymphocytes per 1 mm² tissue were identified and/or if interstitial and/or endothelial expression of CAMs was enhanced. Endothelial expression of HLA-1, HLA-DR and ICAM-1 was semiquantitatively scaled 1 to 3, according to intensity of immunoperoxidase staining of endothelial cells. Endothelial CAM-expression grade 1 represents faint staining (normal), grade 2 represents intense staining, and grade 3 represents abundant immunoreactivity (Fig. 1). As HLA-1, HLA-DR and ICAM-1 are constitutively expressed on endothelial cells, expression was enhanced, if immunoreactivity staining exceeded grade 1. The criteria for endothelial activation were met if, for two or more CAMs, immunoreactivity staining exceeded grade 1. Endothelial activation was graded according to the sum of endothelial expression of HLA-1, HLA-DR and ICAM-1: 3 to 4 normal, 5 to 7 moderate, 8 to 9 abundant. The myocardial biopsies were examined and analyzed by two independent blinded observers.

Endothelial function. Endothelial function of the radial artery was assessed. By means of high-resolution ultrasound, diameter changes in response to reactive hyperemia (flow-mediated vasodilation [FMD]), as compared with glyceroltrinitrate (GTN) (endothelium-independent vasodilation [GTN-MD]), were detected, referring to the standard protocols (26,27). Accuracy and reproducibility have been documented (27); a low coefficient of variation for measurements of arterial diameter and a high correlation between consecutive control measurements have been demonstrated (26). Flow-mediated vasodilation in response to reactive hyperemia (FMD) represents endothelium-dependent vasoreactivity, whereas vasodilation in response to GTN (GTN-MD) indicates smooth muscle cell function and is
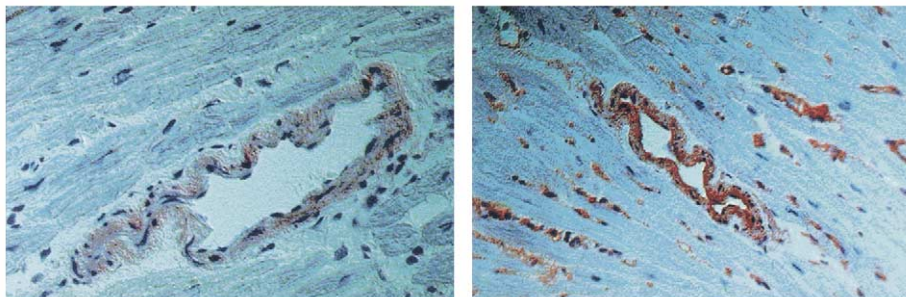
Figure 1. Myocardial biopsies with normal **(left panel)** versus enhanced **(right panel)** expression of endothelial cell adhesion molecules, as determined by immunohistology. Magnification × 400.

independent of endothelial function. Reactive hyperemia, induced by distal cuff occlusion and release, leads to a release of endothelium-dependent vasodilator substances mediated by shear stress, rather than ischemic metabolites. This endothelium-dependent vasodilation can be blocked by the nitric oxide synthase-inhibitor N(G)-monomethyl-L-arginine (23,22). As the vessel diameter after reactive hyperemia is usually maximal after 45 s to 60 s, when flow velocity has already normalized, vasodilation is not due to physical requirements of enhanced flow (13,26,27).

TECHNICAL SETTINGS. The radial artery was examined by two-dimensional ultrasound images, with a 10-MHz linear array transducer and a standard 128XP/10c-system (Acuson, Mountain View, California). The transducer was positioned distal to the elbow to achieve a longitudinal picture of the radial artery. Transmit zone, depth and gain were set to optimize images of the lumen/arterial-wall interface; images were magnified by resolution box function; machine operating parameters were not changed during the study. Diameters were measured by means of a computerized edge detection program (Cardiovascular Imaging Software, Information-Integrity, Boston, Massachusetts); the images were acquired ECG-triggered at end-diastole throughout the study. Arterial flow velocity was measured by pulsed Doppler signal at a 70° angle to the vessel throughout the study.

STUDY PROTOCOL. The subject lay at rest for at least 10 min before beginning the scan for endothelial function. A resting scan was recorded for 1 min. A pneumatic tourniquet, placed at the subject's wrist, was then inflated to a pressure of 300 mm Hg for 3 min. The release immediately induces increased blood flow in the subject's forearm for few seconds, which represents the stimulus for endothelium-dependent vasodilation. Vasodilation is generally maximal after 60 s, when flow has already normalized. The vessel was continuously scanned during the procedure, from baseline until 5 min after release of the cuff. A break of 10 min with the patient continuously supine was required before the scan for endothelium-independent vasodilation was started. After a resting scan, 400 μg GTN was administered sublingually; the scan continued for 5 min after application. Maximal vasodilation generally occurs 4 min to 5 min after GTN administration. One experienced person performed

all scans. The computer-assisted calculation of vessel diameters was conducted in a blinded fashion. The ECG was monitored continuously, and blood pressure was controlled throughout the study. All cardiovascular medication was ceased 12 h to 48 h before the study, depending on half-life.

CALCULATIONS. Flow-mediated vasodilation represents the percentage of diameter increase caused by shear stress, compared with baseline. The GTN-MD represents the percentage of diameter increase induced by GTN, compared with baseline. Flow was calculated from Doppler flow velocity and vessel diameter. Reactive hyperemia was calculated as the maximum flow recorded within the first 15 s after cuff deflation divided by flow during the baseline scan.

Statistical analysis. Statistical analysis was performed with the SPSS Inc. (Chicago, Illinois) software, version 10.0 for IBM-PC. Descriptive data are expressed as mean ± SD. Quantitative data were correlated by use of the Spearman p rank-order analysis, calculating the coefficient of correlation r. Quantitative data were compared to qualitative data by use of the Kruskal-Wallis test on rank sums for independent samples, followed by a post-hoc multiple comparison procedure, if appropriate. To compare quantitative data of two groups, the Mann-Whitney U test was applied. Statistical significance was inferred at $p < 0.01$.

RESULTS

Patients characteristics. Mean age of the 53 patients was 44 ± 13 years; 21 were men, 32 women, mean left ventricular EF was 62 ± 12% (Table 1). All patients were normotensive and had normal lipid levels. Five subjects were treated for hypertension, three for hypercholesterolemia, three were smokers. The patients were on standard cardiovascular medication. There were no significant differences in risk factors or medication between patients at different levels of endothelial activation (Table 1).

Mean age of the 12 control subjects was 45 ± 13 years; six were men, six women, mean EF was 63 ± 14% (Table 1). All patients were normotensive and had normal lipid levels. Two subjects were treated for hypertension, one for hypercholesterolemia, one was a smoker.

Myocardial biopsies. Myocardial inflammation was confirmed by immunohistology in 53 subjects, applying the

Table 1. Patient Characteristics

	Control	MC	Endothelial activation (MC)		
			Normal	Moderate	Abundant
n	12	53	10	26	17
Hypertension (n)	2	5	1	2	2
Hyperlipidemia (n)	1	3	1	1	1
Smokers (n)	1	3	1	1	1
EF (%)	63 ± 14	62 ± 12	67 ± 5	62 ± 11	58 ± 15
Age (yrs)	45 ± 13	44 ± 13	46 ± 11	42 ± 15	46 ± 12
Gender (male/female)	6/6	21/32	4/6	10/16	7/10
d base (mm)	2.40 ± 0.53	2.48 ± 0.46	2.59 ± 0.47	2.47 ± 0.40	2.46 ± 0.54
d max (mm)	2.64 ± 0.56	2.59 ± 0.47	2.78 ± 0.49	2.56 ± 0.41	2.53 ± 0.55
d GTN (mm)	3.08 ± 0.57	3.12 ± 0.48	3.26 ± 0.73	3.11 ± 0.43	3.02 ± 0.34
FMD (%)*	10.11 ± 2.22	4.28 ± 2.72	7.78 ± 2.15	3.74 ± 2.25	3.05 ± 1.92
GTN-MD (%)	28.12 ± 8.37	27.67 ± 8.58	29.91 ± 7.75	27.36 ± 9.75	26.82 ± 7.27

Data is expressed as mean ± SD or absolute numbers (n). For patients with myocardial inflammation (MC), data is depicted for different levels of endothelial activation. No statistically significant difference between different levels of endothelial activation (normal, moderate, abundant) or between MC and control, concerning risk factors, ejection fraction (EF) (p = 0.340/0.441), age (p = 0.430/0.723) or glyceroltrinitrate endothelium-independent vasodilation (GTN-MD) (p = 0.614/0.754). *Statistically significant difference for endothelium-dependent flow-mediated vasodilation (FMD) between different levels of endothelial activation (normal, moderate, abundant) (p < 0.001) and between MC and control (p < 0.001).
d base = diameter of radial artery at baseline; d GTN = diameter of radial artery after GTN; d max = diameter of radial artery after reactive hyperemia.

criteria described above. A total of 12 subjects had normal myocardial biopsies.

Endothelial activation, as demonstrated by enhanced expression of HLA-1, HLA-DR and ICAM-1 in myocardial biopsies, was detected in 43 (81%) of the 53 patients; the extent and the pattern of distribution varied: 5 patients had normal expression levels of HLA-1, HLA-DR and ICAM-1, 6 had enhanced expression of only HLA-1, 7 had enhanced expression of HLA-1 and HLA-DR, 11 had enhanced expression of HLA-1 and ICAM-1, and 24 had enhanced expression of HLA-1, HLA-DR and ICAM-1. The number of patients for each level of endothelial activation is depicted in Table 1.

Endothelial function. GENERAL CHARACTERISTICS. Heart rate (74 ± 8 beats/min) and blood pressure (systolic 121 ± 9 mm Hg, diastolic 74 ± 8 mm Hg) did not change significantly during measurements. Hemodynamic parameters were not significantly different between controls and patients with MC, nor between different levels of endothe-lial activation. Adequate reactive hyperemia as a stimulus for endothelium-dependent vasodilation was achieved in all subjects. Mean resting diameter of the radial artery was 2.47 ± 0.47 mm; after reactive hyperemia the mean diameter increased to 2.60 ± 0.48 mm and after GTN to 3.11 ± 0.49 mm (Table 1).

FMD. Endothelial function, as determined by FMD of the radial artery, in patients with MC, was significantly impaired (FMD$_{MC}$ 4.28%), as compared with controls (FMD$_{Co}$ 10.10%) (Fig. 2) (p < 0.001). The severity of endothelial dysfunction in patients with MC correlates significantly with the extent of endothelial expression of HLA-1 (r = −0.459, p = 0.001), HLA-DR (r = −0.287, p = 0.037) and ICAM-1 (r = −0.385, p = 0.004) in myocardial biopsies (Fig. 3). With increasing, enhanced expression of endothelial CAMs (sum score), endothelial function was progressively impaired, as measured by decreasing FMD (r = −0.433, p = 0.001) (Table 1, Figs. 3 and 4).
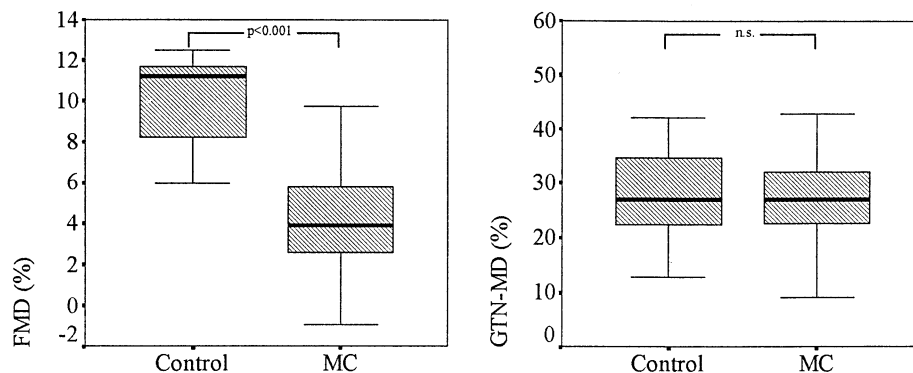
Figure 2. Endothelium-dependent, flow-mediated vasodilation (FMD) **(left panel)** and glyceroltrinitrate endothelium-independent vasodilation (GTN-MD) **(right panel)** in peripheral arteries in patients with myocardial inflammation (MC) as compared with patients with normal myocardial biopsies (Control). Significant difference for FMD (p < 0.001); no significant difference for GTN-MD.
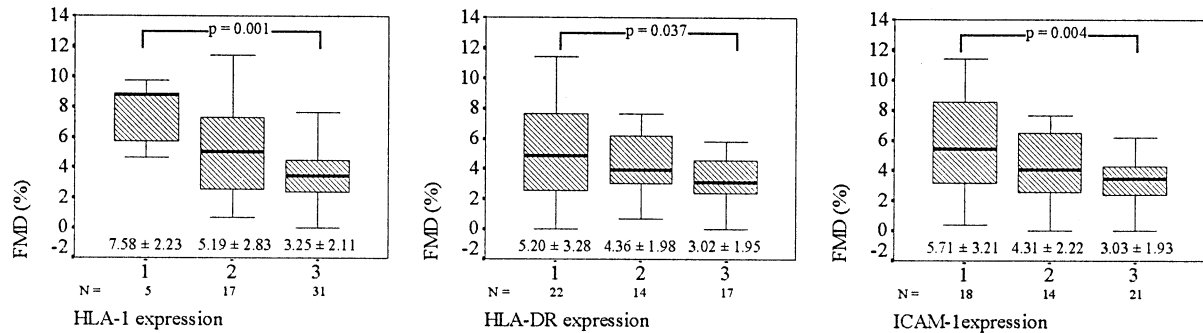
Figure 3. Endothelium-dependent, flow-mediated vasodilation (FMD) in peripheral arteries in relation to endothelial expression of human leukocyte antigen (HLA)-1, HLA-DR and intercellular adhesion molecule (ICAM) in myocardial biopsies of patients with myocardial inflammation. Significant differences (p < 0.05).

GTN-MD. Endothelium-independent vasodilation in patients with MC (GTN-MD$_{MC}$ 27.67 ± 8.58) did not differ significantly from endothelium-independent vasodilation in controls (GTN-MD$_{Co}$ 28.12 ± 8.37) (Fig. 2) (p = 0.754). There was no significant correlation between endothelium-independent GTN-MD of peripheral arteries and the extent of endothelial expression of HLA-1 (r = −0.178, p = 0.202), HLA-DR (r = −0.014, p = 0.922), ICAM-1 (r = −0.085, p = 0.546) or the sum score of CAM expression (r = −0.099, p = 0.480) in myocardial biopsies. **IMPACT OF LEFT VENTRICULAR FUNCTION.** In patients with MC, left ventricular function, as determined by EF in left ventricular angiography, had an effect on endothelium-dependent vasodilation, as well as endothelium-independent vasodilation; there was a significant correlation between FMD and EF (r = 0.414, p = 0.002), as well as GTN-MD and EF (r = 0.299, p = 0.030). However, there were no significant differences in left ventricular function (EF), comparing the different patient groups according to the levels of endothelial activation (Table 1). Additionally,

there was no significant correlation between EF and endothelial activation, as determined by endothelial expression of HLA-1 (r = −0.062, p = 0.660), HLA-DR (r = −0.019, p = 0.895), ICAM-1 (r = −0.261, p = 0.059) and the sum score of endothelial activation (r = −0.174, p = 0.212). **IMPACT OF AGE.** As all subjects in our study population were middle-aged, with only small variations, age had no impact on endothelial function. In patients with MC, there was no significant correlation between age and endothelial function (r = −0.033, p = 0.816) nor between age and endothelial activation (r = −0.097, p = 0.489). There were no significant differences in age comparing the different patient groups according to the levels of endothelial activation (Table 1).

DISCUSSION

The results of this study demonstrate a systemic endothelial dysfunction in patients with immunohistologically confirmed MC. For the first time, endothelial activation in myocardial biopsies has been associated with systemic endothelial dysfunction in patients with MC. The severity of endothelial dysfunction correlates with the extent of endothelial expression of HLA-1, HLA-DR and ICAM-1.

As other factors with impact on endothelial function have been minimized or excluded in this study, we interpret our findings towards a direct interaction between endothelial activation by inflammatory processes and endothelial dysfunction. Endothelium-independent vasoreactivity was not affected by MC or endothelial activation.

The impact of systemic inflammation (6,7,9) or circulating systemic inflammatory markers like C-reactive protein (8) and tumor necrosis factor-alpha (28,29) on endothelial function has been demonstrated for other diseases. Cytokine-stimulated adhesion molecule expression has been observed in cultured human cardiac endothelial cells (30). Similar mechanisms may play a role for endothelial dysfunction in patients with MC. Endothelial dysfunction of peripheral arteries in patients with MC could either be caused by systemic endothelial CAM expression due to generalized vascular inflammation or by circulating cytokines induced by the inflammatory process in the heart.
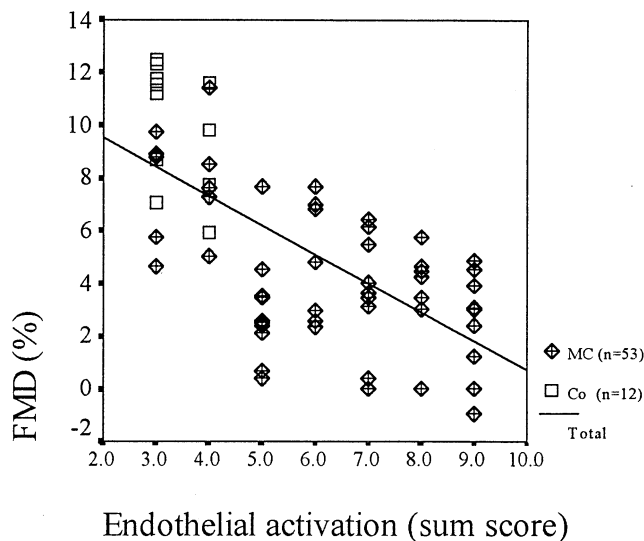
Figure 4. Endothelium-dependent, flow-mediated vasodilation (FMD) in peripheral arteries in relation to endothelial activation (sum score) in myocardial biopsies (Co). Significant correlation (r −0.656, p < 0.001). MC = myocardial inflammation.

A correlation between coronary and peripheral endothelial function has been demonstrated for arteriosclerosis (12). Our recent research is focused on investigating these relations in patients with MC.

Consistent with the literature (17–21,22), we found a correlation between endothelial dysfunction and left ventricular dysfunction. As left ventricular function was equally mildly impaired in all groups with different levels of endothelial activation, this did not alter our results with respect to the effect of endothelial activation and inflammation on endothelial function. Endothelial activation and left ventricular function, both, independently from each other, predict endothelial dysfunction in patients with MC.

To interpret our findings within a clinical context, it is important to understand that endothelial dysfunction potentially represents a marker of prognostic relevance (1,3,4). For patients with ischemic heart disease and after transplantation, this has already been demonstrated (1–5); for patients with MC, it remains to be verified.

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Reprint requests and correspondence: Dr. Katja B. Vallbracht, University Hospital Benjamin Franklin, Department of Cardiology, Free University of Berlin, Hindenburgdamm 30, 12200 Berlin, Germany. E-mail: vallbrac@zedat.fu-berlin.de.

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