Selective Downregulation of VEGF-A165, VEGF-R1, and Decreased Capillary Density in Patients With Dilative but Not Ischemic Cardiomyopathy

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Abstract—Cardiomyopathy (CM) comprises a heterogeneous group of diseases, including ischemic (ICM) and dilative (DCM) forms. The pathogenesis of primary DCM is not clearly understood. Recent studies in mice show that vascular endothelial growth factor (VEGF) is involved in ICM. Whether VEGF plays a role in human CM is unknown. We examined the mRNA and protein expression of VEGF and its receptors in hearts of patients with end-stage DCM and ICM and in healthy individuals using real-time polymerase chain reaction and Western blotting. Number of capillaries, area of myocytes, and collagen were calculated in cardiac biopsies using transmission electron microscopy. In DCM, except for VEGF-C, mRNA transcript levels of VEGF-A165, VEGF-A189, and VEGF-B and the protein level of VEGF-A and VEGF-R1 were downregulated compared with controls (P<0.05). However, in ICM, mRNA transcript levels of VEGF isoforms and protein levels of VEGF-C were upregulated. The vascular density was decreased in DCM but increased in ICM compared with controls (P<0.05). Muscular hypertrophy was not different for ICM and DCM, although DCM had more collagen (P<0.05). Blunted VEGF-A and VEGF-R1 protein expression and downregulated mRNA of the predominant isoform of VEGF-A, VEGF-A165, to our knowledge shown here for the first time, provide evidence that the VEGF-A defect in DCM is located upstream. Whether downregulation of certain VEGF isoforms in DCM is a cause or consequence of this disorder remains unclear, although upregulated VEGF levels in ICM are most likely the result of ischemia. (Circ Res. 2000;87:644-647.)

Key Words: cardiomyopathy • growth factors • angiogenesis

Cardiomyopathy (CM) comprises a heterogeneous group of diseases, including ischemic (ICM) and dilative (DCM) forms. Although several missense mutations have been described in cardiomyocytes of patients with DCM, impairing β-myosin heavy chain gene,1,2 cardiac troponin-T,3 and α-tropomyosin,3 the pathogenesis of primary DCM remains unclear. Recently, it was proven that mice knocked out for vascular endothelial growth factor (VEGF)-A164 and VEGF-A188 had impaired angiogenesis leading to ischemic cardiomyopathy.4 The VEGF isoforms and their receptors are prime regulators of angiogenesis, with overlapping but specific roles in controlling the neovascularization. Tissue oxygen tension tightly regulates VEGF-A levels, because hypoxia rapidly and reversibly induces VEGF-A expression through both increased transcription and stabilization of the mRNA.5,6 Thus, hypoxic upregulation of VEGF-A provides a compensatory mechanism by which tissues can increase their oxygenation through induction of blood vessel growth. ICM is associated with hypoxia, and the results of experiments in mice provide evidence that VEGF-A is a key molecule in regulating the balance between cardiac oxygen consumption and vascular growth. However, whether expression of the VEGF family members might be altered in human DCM is unknown. We examined the gene and protein expression of VEGF and its receptors in cardiac biopsies of patients with DCM and ICM and in samples of nonfailing hearts using real-time polymerase chain reaction (PCR) and Western blotting. We correlated these results to myocardial vascular density, area of myocytes, and collagen. We found a selective downregulation of VEGF-A165, VEGF-A189, VEGF-B, and VEGF-R1 in DCM, whereas protein expression of VEGF-A and VEGF-C was upregulated in ICM. Correlating with this, the vascular density was decreased in myocardial biopsies of patients with DCM but was increased in ICM samples compared with nonfailing hearts.

Materials and Methods

Patients

A total of 41 patients with the diagnosis of heart failure were investigated. Patients gave informed consent to participate in the
studied and were divided into two groups. One group consisted of patients with DCM (n=21) and the other comprised patients with ICM (n=20). Both groups were scheduled for cardiac transplantation and had a routine cardiac transplantation program before surgery. Patients with arterial hypertension, myocardial infarction (6 months before surgery), or echocardiographic evidence of valve disease were excluded from the study. The control group was made up of 10 donors with no history or signs of cardiac disease whose hearts could not be transplanted because of surgical reasons (eg, blood group incompatibility). The results of patients’ blood tests, clinical tests (electroencephalography, echocardiography, and coronary angiography), hemodynamic parameters, and medication were documented. Each patient was given a code and, except for one member of our group who had access to patients’ data, the investigators were unaware of the patients’ case history until the study was finished and the key was broken. Cardiac biopsies of all patients and controls were examined using transmission electron microscopy (TEM), real-time PCR, and Western blotting, as discussed next.

**Real-Time PCR Analysis**

Total RNA was isolated from human heart tissue by a standard guanidinium thiocyanate-phenol-chloroform extraction. cDNA was synthesized using avian myeloblastosis virus reverse transcriptase and 2 μg of total RNA primed with oligo dT-primer. After reverse transcription of RNA into cDNA, real-time PCR was used to monitor gene expression using a Light Cycler instrument (Roche) according to the standard procedure.

**Western Blotting**

The blots were probed with monoclonal or polyclonal antibodies against VEGF-A (Neomarkers), VEGF-B, VEGF-C, VEGF-R2 (Santa Cruz Biotechnology), and VEGF-R1 (Oncogene Research Products) before incubation with horseradish peroxidase-conjugated secondary antibodies (Amer sham Pharmacia Biotech) and exposure to the enhanced chemiluminescence substrate. Blotting reagents were from Amer sham Pharmacia Biotech.

**Criteria and Sites for Obtaining Biopsies**

Multiple myocardial biopsies were taken from the anterior aspect of the left ventricle, according to following criteria. All patients with clear history of myocardial infarction in the anterior cardiac wall (6 months before transplantation) were excluded from the study. There were several selection criteria for final inclusion of the biopsies into the study. To determine if a patient met preoperative criteria, biopsies were taken from prespecified regions of the myocardium. The first diagonal branch of the left anterior descending artery (LAD) was selected for topographical orientation. If the artery was occluded, resulting in myocardial infarction and scar and confirmed by coronary angiography and echocardiography, then the patient was excluded from the study. ICM patients included in the study had stenoses in LAD ranging from 75% to 90% without evidence of infarction (serum enzyme analysis). The biopsy was taken 2 cm distal to the diagonal branch and 2 cm from the LAD. These preoperative criteria helped us to differentiate between necrotic (excluded) and ischemic myocardium (included). Patients were also required to meet histological criteria. If TEM analysis showed unexpected scar regions in the myocardial ultrathin section despite the preoperative tests, then the patient was excluded from the study. Supplementary information about RT-PCR, Western blotting, TEM, and data analysis is available online at http://www.circresaha.org.

### Results

The most important clinical data of the patients and controls are summarized in the Table. Although all parameters in both patient groups (except for age and body surface) were significantly different compared with controls, only two differences were observed between the two patient groups; ie, left ventricular end-diastolic diameter index (LVEDDI) and left ventricular end-diastolic volume (LVEDV) were higher in patients with DCM than in patients with ICM (P<0.05). VEGF-C mRNA and protein expression were significantly increased in both dilative and ischemic CMP forms compared with nonfailing cardiac biopsies (P<0.05). In DCM, mRNA expression of VEGF-A, and VEGF-A protein levels were significantly decreased (P<0.05), whereas in ICM, mRNA (P<0.05) and protein levels (not significant) were increased compared with nonfailing hearts. There was no statistically significant difference between the mRNA and protein expression of VEGF-B or mRNA expression of VEGF-A between the two patient groups, nor were there differences between CMP groups and nonfailing hearts for VEGF-B and VEGF-A. Western blot analysis of VEGF receptors showed that VEGF-R1 (Flt-1) protein expression was decreased in DCM (P<0.05) but was unchanged in ICM compared with nonfailing hearts. VEGF-R2 (KDR/Flk-1) protein expression levels were not different for ICM and DCM compared with nonfailing hearts (Figure).

Analysis of vascular density showed that the number of capillaries in myocardial biopsies of patients with ICM was 58% (26.2±7.4 per area unit) higher (P<0.05), whereas in patients with DCM the number of capillaries was 66% (8.9±6.4 per area unit) lower (P<0.05) compared with nonfailing hearts (16.6±3.8 per area unit). The average area occupied by myocytes was 2207±386 μm²/area unit in ICM, 2326±369 μm²/area unit in DCM (difference not significant), and 1789±218 μm²/area unit in nonfailing hearts (P<0.05). The number of myocytes with degenerated, normal, and edematous mitochondria was equal for ICM and DCM (59%, 36%, and 5%, respectively). The mean cumulative area of collagen was higher (P<0.05) in DCM (270±68 μm²/area unit) compared with ICM (154±32 μm²/area unit).

### Discussion

The development of a normal circulatory system is dependent on normal levels and appropriately timed expression of VEGF-A. It has been demonstrated that targeted disruption of even a single VEGF-A allele in mice results in death of embryos in utero attributable to widespread hemorrhage from...
an inchoate vascular tree. In addition, all mice knocked out for VEGF-A164 and VEGF-A188 developed impaired myocardial contractility, depressed left ventricular relaxation, dilated hearts, subendocardial ischemia, and impaired angiogenesis. Fifty percent of these mice died in utero, and the remainder died within 14 days after birth. These results show that the absence of 2 isoforms of 1 VEGF-A gene seemed to restrict vascularization of myocardium, leading to ischemic cardiomyopathy.

The finding of blunted VEGF-A mRNA and protein expression, associated with reduced levels of its receptor VEGF-R1 (Flt-1), provides evidence that the underlying pathology is located upstream and confirms recent studies suggesting a more important role for VEGF-R1. The differences of VEGF expression between controls and patients with ICM or DCM were subtle; however, over the long term the changes of VEGF may be relevant, because VEGF has a potent gene-dosis effect. Although it remains uncertain whether downregulated VEGF-A gene and protein expression are the cause or consequence of DCM, one could assume that the VEGF pathology associated with reduced capillary density in patients with DCM might lead to impaired muscular contractility. This would explain, at least in part, the worse left ventricular function (LVEDD and LVEDV) in patients with DCM despite the absence of extramural coronary artery obstruction and the lack of difference in muscular hypertrophy between ICM and DCM found in this study. Also in favor of this hypothesis is that mechanical stress increases VEGF expression and, although DCM had the largest LVEDD and LVEDV, VEGF expression was blunted both at mRNA and protein levels in these patients, suggesting a possible signaling pathway for VEGF expression independent of mechanical stress in DCM. Although it remains unclear to what extent the higher amounts of deposited collagen in DCM found in this study could have contributed to downregulated VEGF levels, a different spatial localization of the VEGF forms attributable to a changed extracellular matrix may negatively effect bioavailability and angiogenesis in DCM in addition to the reduced mRNA and protein expression.

Analysis of vascular density in this study showed a significantly higher number of capillaries in ICM, whereas in DCM the number of capillaries was significantly lower compared with nonfailing hearts. These data correspond with the reduced VEGF165 mRNA and protein expression and higher collagen amount in DCM and increased VEGF transcript and protein levels in ICM. Similarly, increased basic fibroblast growth factor and VEGF levels have been reported in the ischemic limb, distally from the occlusion. It seems that coronary stenosis (but not occlusion) leads to a hypoxic stimulus in myocardium that is large enough to induce upregulated VEGF levels and enhanced microvascular angiogenesis in ICM. Therefore, it seems likely that the upregulation of VEGF in ICM is a consequence rather than the cause of the disease. It is difficult to guess the amount of oxygen reaching the cardiomyocytes in light of the matrix issue discussed above. Therefore, future studies should concentrate on correlative studies of the myocardial perfusion, on the one hand, and the intercapillary distance (indicative of oxygen diffusion distance), on the other hand. The fact that ICM developed despite an increased capillary density might be attributable to a pathway yet to be discovered, an impaired responsiveness of these patients to VEGF, or an insufficient level or bioavailability of VEGF. Furthermore, unchanged levels of VEGF-R1 and VEGF-R2 in patients with ICM compared with controls indicate that the reason for development of ICM might be related to downstream events. Evidence is increasing that genetic predisposition factors importantly determine the angiogenic activity of growth factors. Intracoronary administration of human recombinant VEGF-A has been shown to predominantly improve resting but not exercise perfusion, and the delivery of VEGF165 gene improved myocardial perfusion of patients with coronary artery disease. It could be assumed that such patients with
already elevated levels of VEGF may not respond well to VEGF therapy or that they require higher doses of VEGF. mRNA expression levels of VEGF-C were significantly higher for both DCM and ICM. VEGF-C likely plays a dual role as an angiogenic and lymphangiogenic growth factor. Neither VEGF-B nor VEGF-C mRNA levels are regulated by hypoxia. However, it has been shown that VEGF-C is angiogenic in vivo during ischemic conditions. Therefore, it is more likely that elevated VEGF-C mRNA in human CM could affect lymphangiogenesis rather than angiogenesis. The fact that elevated VEGF-C mRNA level does not compensate for the reduced mRNA levels of VEGF-A165, 189 (ie, reduced capillary density in DCM) favors this hypothesis. VEGF-R1 binds VEGF-A but not VEGF-C, whereas VEGF-R2 (Flk-1/KDR) is able to bind both. The reduced protein level of VEGF-A in DCM correlates significantly with reduced expression of Flt-1 in DCM. In contrast, Flk-1/KDR protein levels remained unchanged, although expression of its ligands VEGF-A and VEGF-C were upregulated or downregulated significantly in both ICM and DCM. These data suggest a more important role for VEGF-A and its receptor Flt-1 with respect to the different pathophysiology of DCM versus ICM. Future studies are needed to show whether replacement of the blunted VEGF-A165 could promote the impaired left ventricular function and myocardial angiogenesis in DCM, as shown in this study.

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References
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