Decreased Expression of the Cardiac LIM Domain Protein MLP in Chronic Human Heart Failure

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Background—The cardiac LIM domain protein MLP, a member of the cysteine-rich protein family, is an essential regulator of cardiac muscle development. Mice with a disruption of the MLP gene resemble the morphological and clinical picture of dilated cardiomyopathy and heart failure in humans. We investigated whether altered MLP expression is significant for the pathogenesis of human heart failure.

Methods and Results—Immunohistochemistry and in situ hybridization confirmed the expression of MLP protein and mRNA in human cardiomyocytes. Western blot analysis revealed that the MLP peptide was present in the contractile protein fraction but not in the cytosolic or membrane fraction and that the binding of MLP to myofibrils required functional zinc finger domains. MLP immunoreactivity was decreased 50% (P<0.05) in the left ventricular myocardium of patients with chronic heart failure due to dilated or ischemic cardiomyopathy compared with non-failing donor hearts. MLP mRNA expression, as assessed by Northern blot experiments, was not significantly different between failing and non-failing control hearts, which suggests that decreased MLP synthesis or increased MLP protein turnover, rather than a decreased number of RNA transcripts, may play a role.

Conclusions—Because MLP may promote myofibril assembly, the down-regulation of this adapter protein might play an essential role in myofibril derangement or impaired myofibril rearrangement in the failing human myocardium. (Circulation. 2000;101:2674-2677.)

Key Words: heart failure ■ cardiomyopathy ■ genes ■ RNA

The LIM motif defines a unique, double-zinc-finger structure found in proteins critical to cellular determination and differentiation. Although many zinc-finger motifs bind to specific RNA or DNA sequences, the LIM domain is thought to participate in protein-protein interactions. More than 60 gene products have been identified that display 1 to 5 copies of the LIM domain. They can be classified into the following 3 subclasses: LIM-homeodomain proteins, LIM functional-domain proteins (such as LIM kinases), and LIM-only proteins. The cysteine-rich protein family forms a subclass of the LIM-only proteins. A member of this subclass, the striated muscle-specific LIM-only protein MLP (muscle LIM protein), is a conserved positive regulator of the myogenic differentiation associated with the actin-based cytoskeleton.

In the heart, MLP is expressed at high levels in atrial and ventricular myocytes during fetal development and in the adult. Overexpression of MLP in cultured myoblasts augments differentiation, which suggests that this gene serves as a positive regulator of myogenesis. The importance of MLP for normal cardiac function was demonstrated in mice bearing a targeted disruption of the gene. MLP-deficient animals developed dilated cardiomyopathy with myocyte hypertrophy and heart failure after birth. To understand the role of MLP in normal cardiac physiology and pathophysiology in humans, we analyzed its expression and subcellular localization in left ventricular myocardium derived from explanted human hearts.

Methods

Myocardial Tissue

Failing hearts were obtained from patients undergoing orthotopic heart transplantation due to end-stage heart failure (New York Heart Association class III to IV) resulting from idiopathic dilated cardiomyopathy (n=16; mean age, 51±3 years) or ischemic cardiomyopathy, which was confirmed by coronary angiography (n=8; age, 55±2 years). Medical therapy for the patients had consisted of digitalis, diuretics, nitrates, and angiotensin-converting enzyme inhibitors. None of the patients had received catecholamines. All patients gave written, informed consent before the operation. Myocardial tissue from non-failing hearts that could not be transplanted for surgical reasons or blood group incompatibility were studied for comparison (n=15; age, 43±4 years). The major source of donor hearts was persons dying of spontaneous intracerebral or subarachnoidal bleeding. Neither medical histories nor 2D echocardiography had revealed signs of heart disease in these individuals. Tissue samples from failing and non-failing hearts were snap-frozen in liquid nitrogen no later than 2 hours after explantation.
Immunoblot Analysis
Crude homogenates were obtained by homogenization of left ventricular tissue in 10 volumes of ice-cold buffer (50 mmol/L Tris-HCl [pH 7.5], 5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.1 mmol/L PMSF, and 10 mmol/L EDTA). Cytosol, membranes, and myofibrils were prepared according to standard protocols. To extract myofibrillar proteins sensitive to EDTA, myofibrils were incubated in 50 mmol/L Tris-HCl (pH 7.5) and 10 mmol/L EDTA for 30 minutes at 4°C and centrifuged for 15 minutes at 20,000 g.

SDS-PAGE was performed using a 12.5% gel. Separated proteins were transferred to a nitrocellulose membrane and probed with polyclonal MLP antiserum. This polyclonal antibody against the C-terminal peptide of rat MLP (GGLTHQVEKKE) also recognizes human MLP. The immunoreactive bands were visualized using a chemoluminescence detection system.

Immunohistochemistry
Cryostat sections of left ventricular tissue were fixed in formalin and stained by the indirect streptavidin method. The horseradish peroxidase substrate aminoethylcarbazole was used.

Expression of Recombinant MLP
A cDNA fragment corresponding to the coding sequence of human MLP was generated by polymerase chain reaction and cloned into the pGEX-1T vector. Glutathione-S-transferase (GST)-MLP fusion proteins expressed and purified from clarified bacterial lysate were incubated with a 50% slurry of glutathione agarose beads. Loaded beads were washed 3 times, and GST-MLP was eluted with reduced glutathione and recombinant MLP was separated by thrombin-cleavage.

Blot Hybridization Experiments
Northern blots were prepared from 10 μg of total RNA as described previously. A [32P]-labeled 375-bp fragment of human MLP cDNA was used as a specific probe.

Figure 1. MLP expression in left ventricles from patients with dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) and from non-failing donor hearts (NF). A, Top shows a representative Northern blot probed for MLP and for GAPDH as an internal control. No significant difference in MLP mRNA expression between failing and non-failing hearts was observed. B, Top shows a Ponceau red-stained membrane illustrating equal loading of proteins and a representative Western blot with different concentrations of recombinant MLP (r-MLP) as a positive control. MLP protein concentrations are significantly down-regulated in failing hearts.

In Situ Hybridization
Cryostat sections of left ventricular myocardium were fixed in 4% formaldehyde in PBS and treated with proteinase K. Sense and antisense digoxigenin-labeled RNA probes were prepared by in vitro transcription from a human MLP cDNA insert. Hybridization was performed overnight at 42°C in hybridization buffer (50% formamide, 10% dextran sulfate, 2.5× Denhardt’s, 2.25× SSPE, 100 μg/mL salmon sperm DNA, 5 mmol/L dithiothreitol, and 0.2 μg/mL denatured probe). After high-stringency washes and treatment with RNase A, the sections were stained with an alkaline phosphatase-coupled anti-digoxigenin-coupled antibody and the chromogen 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP).

Statistics
Data are expressed as mean±SEM. Statistical significance was estimated with 1-way ANOVA and the Bonferroni t test. P<0.05 was considered significant.

Results
MLP is Down-Regulated in Chronic Heart Failure
To explore the possibility that altered MLP expression may play a role in the pathogenesis of human heart failure, MLP mRNA and protein concentrations were determined in left ventricular myocardium. Northern blot experiments (Figure 1A) revealed no significant difference in steady-state MLP mRNA concentrations in non-failing donor hearts and in failing hearts from patients with both dilated and ischemic cardiomyopathy (non-failing: 1.9±0.6 MLP mRNA/GAPDH mRNA, n=15; dilated cardiomyopathy: 0.9±0.1 MLP mRNA/GAPDH mRNA, n=16; ischemic cardiomyopathy:...
0.9±0.2 MLP mRNA/GAPDH mRNA, n=8). However, a marked variability was observed in the non-failing group. In contrast to the Northern blot data, Western blot experiments (Figure 1B) demonstrated decreased MLP protein concentrations in diseased myocardium (non-failing: 0.35±0.04 µg of r-MLP equivalent, n=14; dilated cardiomyopathy: 0.16±0.02 µg of r-MLP equivalent, n=14, P<0.05 versus non-failing hearts; ischemic cardiomyopathy: 0.13±0.03 µg of r-MLP equivalent, n=8, P<0.05 versus non-failing hearts).

MLP Is Associated With Myofibrils
To investigate the cellular localization of MLP, immunohistochemical staining of cryostat sections of human left ventricular myocardium derived from donor hearts and from explanted hearts with dilated or ischemic cardiomyopathy was performed. The specific MLP antiserum detected a cytoplasmic antigen in cardiomyocytes in a mainly striate pattern (Figure 2A). Furthermore, MLP immunoreactivity accumulated at the cell periphery. MLP was obviously not localized to nuclei because nuclear staining was absent, even when fixative or fixation time were changed. Consistent with immunocytochemistry findings, in situ hybridization using a specific MLP riboprobe revealed strong cardiomyocyte staining (Figure 2B).

MLP Binding to Myofibrils Requires Functional Zinc Finger Domains
In an effort to investigate the subcellular distribution of MLP protein, protein fractions containing membrane proteins, cytosolic proteins, or myofibrillar proteins were analyzed for MLP expression. These experiments demonstrated that MLP immunoreactivity accumulates in the fraction containing contractile proteins (Figure 2C). If the binding of MLP requires the presence of coordinated zinc in the LIM motif, the MLP-myofibrill association should be sensitive to EDTA. Indeed, MLP was enriched in the EDTA-soluble protein fraction after the incubation of myofibrils with 10 mmol/L divalent metal chelator (Figure 2D). Furthermore, the MLP present in the myofibril extract from the left ventricular myocardium binds to recombinant GST-MLP immobilized with glutathione sepharose (Figure 2E). This binding was reduced in the presence of EDTA.

Discussion
Data from the present study demonstrate that the muscle LIM domain protein MLP, expressed in the human left ventricular myocardium, is down-regulated in end-stage heart failure, regardless of the cause (either ischemic or dilated cardiomyopathy).

Recent studies using animal models suggest an important role for MLP, both in the developing and in the adult heart. During mouse embryogenesis, expression of MLP occurred at embryonic day 9 in the developing atrial and ventricular heart chambers and it remained at high levels through the end of gestation and into adulthood.7 Disruption of the MLP gene in mice leads to progradient alterations in the cytoarchitecture of cardiac muscle cells. Because the MLP knockout animals reproduce the clinical and morphological picture of dilated cardiomyopathy and heart failure in humans, it has been hypothesized that altered expression of MLP might play a role in the pathogenesis of human heart failure.
To address this question, we investigated MLP expression in the failing left ventricular myocardium from patients with dilated and ischemic cardiomyopathy. MLP protein concentrations were significantly decreased in failing hearts, whereas MLP mRNA concentrations were unchanged compared with non-failing donor hearts. These results indicate that decreased MLP synthesis or enhanced MLP protein turnover, rather than a decreased number of MLP transcripts, may play a critical role in human heart failure. Interestingly, these alterations are not confined to dilated cardiomyopathy. They can also be detected in hearts dilated due to coronary heart disease.

It has been suggested that MLP is important for the organization of actin-based structures and the regulation of cardiomyocyte cytoarchitecture.4,8 These studies, however, all referred to animal experiments. Our data now demonstrate that MLP is also associated with myofibrils in the human myocardium and that its binding requires the presence of Zn$^{2+}$ in the LIM motif. Our results from immunohistochemistry studies are in line with the suggestion that MLP is associated with lateral myofibril anchorage sites and Z-lines of myofibrils.4 In chronic heart failure in humans, ultrastructural alterations, such as a disruption of myofibrillar organization, a rarefaction of sarcomeres, and an increase in nonmyofibrillar space, have been identified as morphological correlates of reduced myocardial function.9 The results from the present study suggest that reduced MLP content might promote the deterioration of the contractile function of the myocardium afflicted with dilated or ischemic cardiomyopathy. The discovery of this cellular alteration could provide a target for novel therapeutic interventions.

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**References**

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