RELATION BETWEEN MYOGLOBIN AND CARDIAC DYSFUNCTION IN MYOCARDITIS

—Immunohistochemical Study of Endomyocardial Biopsy Specimens—

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To investigate the mechanism of cardiac dysfunction in myocarditis, myoglobin, an intracellular oxygen-transport, was immunohistochemically examined in biopsy specimens obtained from the right side of the ventricular septum and left ventricular free wall in 58 patients with myocarditis and 19 controls.

Sections 4 μ m thick were stained by the indirect immunoperoxidase method using a polyclonal antibody to human myoglobin as the primary antibody. Under light microscopy, the intensity of myoglobin immunoreactivity in the tissue section was semiquantitatively classified from grade 0 to grade 3. Then, the grade of myoglobin staining was compared with clinical, hemodynamic and histopathologic parameters.

In right and left ventricular specimens, the grade of myoglobin staining was positively correlated with ejection fraction, but inversely with left ventricular end-diastolic and end-systolic volume indices. The percentage of myocytes with grade 0 was correlated with the number of mononuclear cells in the specimens. In addition, the grade of myoglobin staining in right ventricular specimens was positively correlated with the duration of illness but inversely correlated with the number of mononuclear cells. In 4 patients who had serial biopsies, the ejection fraction was improved and the grade of myoglobin staining was increased in the convalescent stage.

These results indicate that myoglobin staining reflects the intensity of myocarditis and a decrease of myoglobin may be important as one of the pathogenetic factors of cardiac dysfunction in myocarditis.

MYOGLOBIN is a low-molecular weight (17,500 daltons) oxygenbinding protein that is present in both striated skeletal and cardiac muscle cells! It has a higher affinity for oxygen than hemoglobin, and is

important in intracellular oxygen transports?

Kagen et al reported that myoglobin was released from cardiac muscle and rapidly cleared from the circulation following acute myocardial infarction³. The detection of myoglobin in blood and urine is valuable for the diagnosis of acute myocardial infarction. Recently, we reported that myoglobin disappeared at the early stage of acute myocar-

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TABLE I THE PATIENTS WITH MYOCARDITIS AND CONTROLS (mean $\pm$ SD)

Group		n	Age (yr.)	Sex (M/F)	Duration of illness(w)
Myocarditis	RVB	36	41 $\pm$ 15	22 / 14	6 $\pm$ 4
	LVB	22	42 $\pm$ 17	13 / 9	10 $\pm$ 5
Control	RVB	13	47 $\pm$ 17	9 / 4	(-)
	LVB	6	46 $\pm$ 22	4 / 2	(-)

Abbreviations: RVB, LVB=right and left ventricular endomyocardial biopsy specimens.

*Seventeen patients in myocarditis and three patients in control had both RVB and LVB.

dial infarction in formalin-fixation-paraffin-embedded tissue by the immunohistochemical method using myoglobin antibody^{4,5}

Myocarditis sometimes progresses to congestive heart failure following myocardial cell necrosis, degeneration and/or interstitial edema⁵⁻⁹. Ischemia due to microcirculatory collapse and/or direct cellular damage due to viral infarction are considered as the pathogenesis, but the details are unknown.

Thus, to clarify the pathogenesis of cardiac dysfunction in myocarditis, myoglobin was immunohistochemically determined using endomyocardial biopsy specimens and was compared to hemodynamic data and inflammation in patients with myocarditis.

MATERIALS AND METHODS

Patient Profile

Endomyocardial biopsy specimens of 77 patients were studied. There were 48 men and 29 women ranging in age from 18 to 65 years; 58 with myocarditis and 19 controls. There were no significant differences in age or sex between patients with myocarditis and control groups (Table I).

Diagnostic definition of myocarditis is as follows.

1) Clinically, patients must show ST-segment and T wave changes, ventricular arrhythmia and complete atrioventricular block on the electrocardiogram and/or symptoms of dyspnea, palpitation, precordial discomfort and chest pain following "flu-like" symptoms such as fever, myalgia, malaise and arthralgia⁹.

2) Upon endomyocardial biopsy, the mean number of mononuclear cells per field at a magnification of 400 was over 5 and/or

max was over 10⁹.¹⁰ Of the 58 patients with myocarditis, 42 patients had both 1) and 2), 11 patients had only 1) and 5 patients had only 2).

The control group consisted of patients who were clinically suspected of having some cardiac disease because of slight chest pain, minimal ECG change or arrhythmia, but for whom the invasive examinations of coronary angiography and biopsy findings were not diagnostic.

Hemodynamic and Angiographic Evaluation

Right and left heart catheterization were done using the standard techniques. The heart rate and pressure from the right and left heart were recorded, and the cardiac index (CI) was estimated by the thermodilution method. Left ventricular end-diastolic and end-systolic volume indices (LVEDVI and LVESVI) and ejection fraction (EF) were calculated from the left ventricular cineangiogram performed in the right anterior oblique projection by Kennedy's method¹¹. Furthermore, left ventricular high-fidelity pressure measurements were obtained in 9 patients (7 with myocarditis and 2 controls) by means of a Millar 7F-micromanometer-angio catheter during left ventriculography. In these patients, peak systolic and end-diastolic circumferential wall stress (Sps and Sed) were calculated as previously reported^{12,13}.

Endomyocardial Biopsy Procedure and Histologic Evaluation

Biopsy specimens were obtained during cardiac catheterization. They were taken from both the right ventricular side of the ventricular septum (RVB) and left ventricu-

Fig.1. Immunohistochemical preparation of tissue obtained at ventricular biopsy. ($\times 200$)

(A)–(D): The myocytes of myoglobin staining grade 0–3.

(A): grade 0; (B): grade 1; (C): grade 2; (D): grade 3

(E)–(H): Comparison of histology between myoglobin staining and hematoxylin-eosin staining in acute myocarditis.

(E) and (F): Marked myocarditis. In hematoxylin-eosin stain (F), there are numerous inflammatory cell infiltrations, edema and degeneration of myocytes. In the serial section of (F), the degree of myoglobin by immunohistochemical method was variable from cell to cell; myocardial cells with grade 0 to 3 myoglobin staining were mixed, as shown in (E). The features of low grade myocytes were localization of myoglobin into the cell membrane. (E & F: $\times 400$)

(G) and (H): Mild myocarditis. Immunohistochemical method using myoglobin (G) and for the same area of the serial section (G) by hematoxylin-eosin stain. (G & H: $\times 400$) Numerals indicate grade of myoglobin staining in the myocytes.

lar free wall (LVB) by Konno-Sakakibara¹⁴ or Kawai¹⁵ The biopsy specimens were immediately fixed with 10% buffered formalin

solution, embedded with paraffin and cut serially into 4 μ m thickness. These were stained with hematoxylin-eosin and Masson's

TABLE II COEFFICIENT OF CORRELATION BETWEEN THE GRADE OF MYOGLOBIN STAINING IN THE BIOPSY SPECIMENS AND CLINICOPATHOLOGIC VARIABLES

	Grade of myoglobin staining			
	RVB	(n)	LVB	(n)
<i>Clinical data</i>				
Duration of illness	0.46*	(31)	0.31	(18)
EF	0.50**	(40)	0.57**	(25)
CI	0.02	(45)	-0.06	(24)
HR	0.15	(45)	0.25	(24)
PCWP	-0.09	(45)	-0.04	(24)
RVSP	-0.04	(45)	-0.05	(24)
RVEDP	-0.05	(45)	-0.04	(24)
LVSP	0.18	(40)	0.21	(25)
LVEDP	-0.17	(40)	-0.11	(25)
LVEDVI	-0.46**	(40)	-0.75**	(25)
LVESVI	-0.55**	(40)	-0.75**	(25)
Sps	-0.30	(10)	0.32	(10)
Sed	-0.22	(10)	0.22	(10)
<i>Pathologic data</i>				
Size of myocytes	-0.24	(49)	-0.27	(28)
Number of mononuclear cells	-0.37*	(49)	-0.05	(28)

Abbreviations: CI=cardiac index; EF=ejection fraction; HR=heart rate; LVEDVI/LVESVI=left ventricular end-diastolic and end-systolic volum indices; LVSP/LVEDP=left ventricular peak systolic and end-diastolic pressure; PCWP=pulmonary capillary wedge pressure; RVSP/RVEDP=right ventricular peak systolic and end-diastolic pressure; Sps/Sed=peak systolic and end-diastolic circumferential wall stress; RVB, LVB=right and left ventricular endomyocardial biopsy specimens.

Significance: *, $p < 0.05$, **, $p < 0.01$

trichrome.

Immunohistochemical Procedure

The peroxidase-antiperoxidase technique was used^{16,17} Deparaffined sections were soaked in absolute methanol containing 0.3% hydrogen peroxide for 15 min at room temperature in order to block the endogenous peroxidase activity. After washing in cold running tap water and phosphate buffer saline (PBS), the sections were exposed to 1:20 diluted normal swine serum (DAKO-immunoglobulins Ltd., Denmark) for 15 min at room temperature. Sections were then incubated with the anti-myoglobin polyclonal immunoglobulin (DAKO) at a dilution of 1:1000 over night at 4°C. They were then washed with PBS, and treated with anti-rabbit IgG swine serum (at a dilution of 1:20, DAKO), and peroxidase-antiperoxidase (PAP) solution (at a dilution of 1:80,

DAKO), for 30 min each, at room temperature. Finally, the sections were soaked in a pH 7.6 0.05M Tris-HCl buffer, containing 3, 3'-diaminobenzidine hydrochloride (40 mg/100 ml) and hydrogen peroxide (0.0015%) for 3 min and counterstained with Mayer hematoxylin.

Classification of Histological Findings

Under a light microscope, myoglobin immunoreactivity in each tissue section was evaluated semiquantitatively. The intensity of immunostaining was graded as follows: 0 (none), 1 (mild), 2 (moderate) and 3 (marked) (Fig. 1, A-D). All myocytes were counted, and their average defined as "grade of myoglobin staining." In each specimen, 20 to 241 myocytes were observed (mean 67). All specimens were graded in comparison with myocytes of control specimens, which were stained together. Speci-

Fig.2. Relation between grade of myoglobin staining and ejection fraction. RVB, LVB; right and left ventricular endomyocardial biopsy specimens. Open circle; myocarditis, closed circle; control.

Fig.3. Relation between grade of myoglobin staining and duration of illness. RVB, LVB; right and left ventricular endomyocardial biopsy specimens. Open circle; myocarditis, closed circle; control.

mens stained by replacing the myoglobin antibody with PBS were used as negative controls. Margin of the specimen, compressed area due to biopsy and transverse sectioned myocyte were not counted in order to exclude artifact. All sections were observed without any prior information and graded independently by two morphologists (KH and HF). The variability between these observers in each specimen was 0.2 ± 0.1 .

Statistical Analysis

Clinicopathologic data were expressed as mean $\pm$ standard deviation (SD). Statistical comparisons were performed using correlation coefficient, Student's *t* test and one-way analysis.

RESULTS

Inflammation and Myoglobin Staining

In the tissues with marked inflammatory changes indicated by numerous inflammatory cell infiltration, interstitial edema and degeneration of myocytes, the degree of myoglobin was variable from cell to cell; myocardial cells with grade 0 to 3 myoglobin staining were mixed (Fig. 1, E). The features of low-grade myocytes were margination of the myoglobin into the cell membrane. In many of the myocytes, the abnormality was not noted by hematoxylin eosin stain (Fig. 1, E–H).

Relation between Grade of Myoglobin Staining and Clinicopathologic Parameters

Table II shows the coefficients of correlation between grade of myoglobin staining in the right- or left-sided biopsy specimens and various clinicopathologic parameters.

The grade of myoglobin staining correlated positively with EF (in RVB and LVB)

Fig.4. Relation between grade of myoglobin staining and left ventricular volume index. LVEDVI, LVESVI; left ventricular end-diastolic and end-systolic volume indices. RVB, LVB; right and left ventricular endomyocardial biopsy specimens. Open circle; myocarditis, closed circle; control.

Fig.5. Relation between grade of myoglobin staining and the number of mononuclear cells. HPF; high power field. RVB, LVB; right and left ventricular endomyocardial biopsy specimens. Open circle; myocarditis, closed circle; control.

and duration of illness (in RVB). It also correlated inversely with LVEDVI (in RVB and LVB), LVESVI (in RVB and LVB) and the number of mononuclear cells (in RVB) (Fig. 2–5). There was also a correlation between the number of mononuclear cells and percent of grade 0 staining cells (in RVB and

LVB) (Fig. 6).

In the same heart, the grade of myoglobin staining was similar between LVB and RVB (Fig. 7).

The duration of illness correlated inversely with the number of inflammatory cell infiltrations ($r=0.42$, $p<0.05$ in RVB, $r=0.38$,

Fig. 6. Relation between percent of myocytes with myoglobin staining of grade 0 and the number of mononuclear cells. HPF; high power field. RVB, LVB; right and left ventricular endomyocardial biopsy specimens. Open circle; myocarditis.

Fig. 7. Comparison of grade of myoglobin staining between left and right ventricular endomyocardial biopsy specimens in the 20 patients examined. RVB, LVB; right and left ventricular endomyocardial biopsy specimens. Open circle; myocarditis, closed circle; control.

$p < 0.05$ in LVB) and positively with EF ($r = 0.44$, $p < 0.05$ in RVB, and $r = 0.40$, $p < 0.05$ in LVB).

In four cases, biopsies were serially done in the acute stage (within 3 weeks after the onset of myocarditis) and the convalescent stage (3 to 24 weeks after the onset of myocarditis). In all four cases, EF showed improvement and myoglobin staining grade was increased at the convalescent stage (Fig. 8).

Fig. 8. Relation between grade of myoglobin staining and ejection fraction in serial biopsies in the acute stage (within 3 weeks after the onset of myocarditis) and the convalescent stage (3 to 24 weeks after the onset of myocarditis). In each of them, EF showed improvement and grade of myoglobin staining increased at the convalescent stage. RVB, LVB; right and left ventricular endomyocardial biopsy specimens. Open circle; first biopsy (acute stage), open triangle; second biopsy (convalescent stage).

DISCUSSION

The present study revealed that the grade of myoglobin staining correlated positively with EF as an indicator of contractility but not with Sps and Sed as indicators of

hemodynamic burdens. The grade of myoglobin staining correlated inversely with the number of inflammatory cell infiltrations and positively with the duration from the onset of myocarditis. The duration of illness correlated inversely with the number of inflammatory cell infiltrations and positively with EF. These indicate that, in acute myocarditis, myoglobin in the myocytes decreases in the acute stage and is restored in the healed stage. Decreasing of myoglobin is not dependent on the abnormality of hemodynamic burden, but depends on the severity of inflammation. It is well-known that the decrease of myoglobin causes hypoxia in the muscle cells and decreases the contractility, because myoglobin transports oxygen from myocardial cell membranes to the mitochondria^{4,5,18-24}. Therefore, a decrease of EF in myocarditis may be related with a decrease of myoglobin.

When contractility of myocytes, an indicator of which is EF, decreases, the various compensatory mechanisms of increased LVEDVI (Frank-Starling's law) and heart rate, hypertrophy of myocytes, etc., appear, and CI (EF $\times$ heart rate) and LVEDP are normalized by them²⁵. In the present study, the grade of myoglobin staining correlated with LVEDVI, but not with CI and LVEDP. This discrepancy may be explained by the fact that CI and LVEDP were normal by compensatory mechanisms in most of the patients in whom endomyocardial biopsy was performed.

A clear histological feature of the myocytes with low-grade myoglobin staining was margination of myoglobin into the cell membrane. This shows that in myocarditis, the cell membrane of the myocyte is damaged and myoglobin released from the myocytes. It is unknown whether the production of myoglobin decreases at the same time. In the normal controls, the grade of myoglobin staining was 2.2 ± 0.2 . None of the myocytes belonged to grade 0, 10% belonged to grade 1, 60% to grade 2 and 30% to grade 3. Therefore, grade 0 probably indicates irreversible cellular damage. However, it is unclear whether grade 1 indicated reversible or irreversible cellular damage. This also indicates that a deviation of myoglobin from the myocyte occurs even in reversible cellular damage.

One of the limitations of this study is that the margin of the specimen and the compressed area cannot be counted because they are almost always grade 0. Transversely sectioned myocytes were excluded, since myocytes in biopsy specimens have the contraction band and intensity of myoglobin staining in a myocyte was different between areas with and without contraction bands. Because of this, it was impossible to determine the grade of myoglobin in transversely sectioned myocytes.

In this study, two trained observers determined the grade of myoglobin staining without prior knowledge of the specimens. There were no significant differences between their decisions ($r=0.88$, $p<0.01$). There were no significant differences between the first staining and second staining with the same specimens ($r=0.91$, $p<0.01$). Therefore, the morphometric data on myoglobin is reliable.

The correlations were statistically significant between grade of myoglobin staining and various clinical or morphologic parameters. However, some variant specimens were also seen in this study. These may be attributed to the fact that the biopsy specimens were too small to represent the whole ventricle.

In conclusion, in myocarditis, a decrease of myoglobin in myocytes occurs with the severity of inflammation and is one of the pathogenetic factors for depressed contractility of myocytes.

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