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Alteration of Antioxidants During the Progression of Heart Disease in Streptozotocin-Induced Diabetic Rats

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Involvement of oxidative stress is implicated in the progression of complication of diabetes mellitus. With respect to heart diseases, we have studied role of oxidative stress/antioxidants using rats treated with streptozotocin to induce diabetes (DM). Hemodynamic and echocardiographic measurements showed thickening of the wall and an increase in the internal dimension of the left ventricle (LV) in DM rats at 8th week. Decrease in diastolic posterior wall velocity and rate of LV pressure change, and increase in LV end diastolic pressures also proved cardiac dysfunction. These changes were further developed in DM rats after 12 weeks. Utilizing rat hearts at 8th and 12th weeks, the following estimations were performed. There was a decrease in the activity of Mn-superoxide dismutase (SOD), suggesting abnormal mitochondrial metabolism of reactive oxygen species. The level of glutathione (GSH) decreased concomitant with a decrease in the expression of γ-glutamylcysteine synthetase (γ-GCS). The expression of transforming growth factor-β1 (TGF-β1), known as a growth factor and a suppressor of GSH synthesis, elevated in DM rat hearts. Immunohistochemical estimation showed an increase in type IV collagen in DM hearts. Collectively, it was suggested a linkage between mitochondrial damage to generate reactive oxygen species and inactivation of Mn-SOD and elevation of the expression of TGF-β1 to lead suppression of GSH synthesis and induction of fibrous change for the consequent cardiac dysfunction in DM.

Keywords: Diabetes, cardiac function, glutathione, Mn-superoxide dismutase

INTRODUCTION

Chronic congestive heart failure is a common and serious complication of diabetes mellitus (DM)[1]. Although usually ascribed to the effects of obstructive extramural coronary artery disease[2], myocardial dysfunction was recently reported to be associated with DM in the absence of extensive coronary artery atherosclerosis[3]. The exact mechanism is not still unknown, however, several findings have been proposed including microvas-

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cular disease, metabolic disorders, and interstitial fibrosis. Most clinical studies of the diabetic heart in failure have the marked diminution of ventricular function[4], which is characterized by impairment of left ventricular (LV) relaxation and systolic performance[5].

Reactive oxygen species (ROS) and an imbalance of ROS/antioxidants are implicated in many pathological states including DM. For example, activated vascular phagocytes produce ROS to induce cellular damage[6]. The levels of ROS are controlled by various cellular defense mechanisms consisting of enzymatic and nonenzymatic scavenger components[7]. Exacerbation of ROS has been reported in uncontrolled DM as manifested by marked alterations in tissue antioxidant enzyme activities. It is strongly suggested that ROS and tissue antioxidant status are an important factor in the etiology of DM and its complications[2, 8, 9].

Mammalian cells contain the copper-zinc form of superoxide dismutase (Cu,Zn-SOD) in cytoplasm and the manganese form (Mn-SOD) in mitochondrial matrix. Mn-SOD is known to be induced by cytokines[10] and one of the most important factors in combating the cytotoxicity of tumor necrosis factor-α (TNF-α), ionizing radiation, and hyperthermia[11].

Glutathione (GSH) plays an important role in scavenging ROS and regulating intracellular redox cycle. GSH is synthesized by two ATP-requiring enzymes, γ -glutamylcysteine synthetase (γ-GCS) and GSH synthetase. γ-GCS is composed of a heavy subunit (γ-GCSh), a catalytic domain, and a light subunit, a regulatory domain, and catalyzes the rate-limiting step of GSH synthesis[12]. We have reported the presence of low GSH concentrations in erythrocytes from diabetic patients[13], endothelial cells from experimental diabetic rabbits[14], and diabetic embryos[9,15]. Furthermore, we have shown that the expression of γ-GCS mRNA is reduced specifically in diabetic conditions[9,16].

However, the possible contribution of the alterations of antioxidants is not clear to the process of cardiac dysfunction in DM. Therefore, we investigated; [1] whether the activity and protein and gene expressions of antioxidants are altered in DM rat hearts, and if so, [2] whether such alterations are related to the impaired cardiac function in DM rats.

MATERIALS AND METHODS

Materials

Using six-week-old male Sprague-Dawley rats (Charles River Japan Inc, Kanagawa, Japan) weighing 175–200g, DM was induced with 65 mg/kg-body weight of streptozotocin (STZ) (The Upjohn Company, Kalamazoo, Mich.) dissolved in 0.5 ml of 0.9% saline with 0.05 M sodium citrate, pH 4.5. Injections were made intraperitoneally in fasted animals. Blood glucose was determined at 4 weeks after STZ injection. The rats whose blood glucose was greater than 250 mg/dl, when tested with glucose oxidase reagent strips (Dextrostix, Ames) were defined as DM rats. Overt DM was ensured 3 days after injection. Acetonuria was uniformly absent in the DM rats. Two groups of animals were studied: 12 for controls, 12 for DM. The experimental period was 8 weeks and 12 weeks of documented DM, and all animals were the same age at the time of sacrifice.

Echocardiographic analysis of cardiac performance

Eight weeks and 12 weeks after induction of DM, M-mode and two-dimensional echocardiography were performed under diethyl ether anesthesia using a Toshiba SSA-380A (Toshiba, Tokyo, Japan) with a 10 or 7.5 MHz transducer. The transducer was calibrated with phantoms before use. Two-dimensional echocardiograms were recorded from parasternal long and short-axis views. M-mode recordings were obtained of the LV at the level of the mitral valve

in the parasternal view using two-dimensional echocardiographic guidance in both the short- and long-axis views. Echocardiographic measurements included M-mode interventricular septal thickness (IVS), LV internal dimension at the end of diastole and systole, and M-mode posterior LV wall thickness (PW) using the leading edge-leading edge method as recommended by the American Society of Echocardiography[17].

Hemodynamic measurements

At the conclusion of the study period, the animals were weighed, anesthetized with diethyl ether, and measured for direct LV pressure (LVP) and the positive and negative rate of change of LVP (+LV dP/dt and -LV dP/dt) through a polyethylene catheter (PE50) advanced to the LV via the right carotid artery with a P50 Statham pressure transducer (Gould Inc, Puerto Rico) and AP-601G and EQ-601G polygraph (Nihon Koden, Tokyo, Japan). The measurements were recorded with a WT-647G recorder (Nihon Koden, Tokyo, Japan). Then, the plasma glucose concentration was determined by the glucose-oxidase H_2O_2 electrode method (BM Blood Sugar, Boehringer Mannheim, Tokyo, Japan). All blood collections were made in the afternoon between 3 and 5 PM. At the end of the experiment, the hearts were rapidly removed from the chest and immersed in ice-cold PBS ([in mmol/L] KCI 2.7, KH_2PO_4 1.5, NaCl 137, and Na_2HPO_4 8.1, pH 7.0). The hearts were immediately frozen in liquid N_2 and stored at −80°C until use.

Estimation of superoxide dismutase activity

The activity of SOD was estimated photometrically as described by Beutler *et al.*[18], based on the inhibition of nitroblue tetrazolium reduction by the O_2 produced via photoreduction of riboflavin. A 50% inhibition was defined as 1 unit of SOD activity. The activity of Mn-SOD was estimated in the presence and absence of potassium cyanide. The activity not inhibited by potassium cyanide was expressed as Mn-SOD activity.

Estimation of GSH

After homogenization of the excited left ventricles in 5 vol. of ice-cold phosphate buffered saline (9 vol. of 0.154 M NaCl and 1 vol. of 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4) in a Polytron homogenizer (Kinematica AG, Switzerland), the homogenate was centrifuged at 13,000 × g for 10 min. The supernatant was stored at −80°C until use. The level of GSH was estimated enzymatically as described by Beutler *et al.*[18].

Northern blots

The probes used were as follows: (a) a γ -GCSh probe (768 bp), (b) a Cu,Zn-SOD probe (0.7 kb), (c) a Mn-SOD probe (1.0 kb)[19], (d) a transforming growth factor β-1 (TGF-β1) probe (604 bp). Rat cDNA probes for TGF-β1 was cloned by polymerase chain reaction using 5′-GAAACG-CAAGCGCATCGAAGCCT-3′ as a 5′ primer and 5′-GATCAGTCCCAAACGTCGAGGTG-3′ as a 3′ primer. This primer corresponds to region that is highly conserved among the different forms of TGF-β. These probes were radiolabeled with ^{32}P using Random Primer DNA Labeling kit (Takara Co. Ltd., Tokyo Japan). The isolation of cytoplasmic RNA and Northern blotting were carried out essentially as described by Sambrook *et al.*[20]. Cytoplasmic RNAs isolated from cells were subjected to electrophoresis in 1% agarose gels containing 0.6 M formaldehyde, subsequently transferred to nylon membranes, and then hybridized with ^{32}P-labeled probes. Autoradiographed membranes were analyzed using a Fujix Bio-Analyzer BAS-5000 (Fuji Photo Film, Tokyo, Japan). After stripping, the membranes were rehybridized with ^{32}P-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe and the intensity of the bands was estimated. The relative radioactivity was expressed

as a ratio of photostimulated luminescence (PSL) corrected by the intensity of GAPDH.

Western blots

The samples were prepared from myocardium preserved at-80°C. The lysate (9 μg) was separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunologically stained using rabbit polyclonal antibody against Cu,Zn-SOD, and Mn-SOD[22]. The bound antibodies were made visible with an alkaline phosphatase-coupled second antibody using a Proto Blot kit. The protein concentration was determined according to Redinbaugh and Turley[23] with bovine serum albumin as the standard. The resulting image was saved in the program NIH-Image. Mean density was calculated from a calibration of the Kodak scale using measured "step" values vs. the given "step" values and converting this from the grey scale to optical density units using Rodbard, an equation which takes into account that the conversion is not linear. This equation is part of the NIH-image program. Data was exported to a StatView (Abacus) spread sheet.

Immunohistochemistry

Paraffin-embedded samples from LV subendocardial area were sectioned into 3 micronmeter and subjected to indirect immunohistochemical staining. The sections were pretreated with 1% pepsin for 60 min at 37°C, and then washed with PBS. After exposed to 4% skin milk at room temperature to block nonspecific staining, the sections were incubated with anti-type IV collagen antibody (Shiseido Company, Tokyo, Japan) overnight at 4°C. The primary antibody was used at the dilution of 1/1000.

After washed with PBS, the sections were incubated with biotin-conjugated second antibody (1/200) at room temperature for 30 min. Then the sections were incubated with alkaline phosphatase-conjugated streptavidin at room temperature for 30 min. Immunostains were visualized by reaction with a solution of new fuchsin. Counter staining was performed using Mayer's hematoxylin.

Statistical analysis

The data are given as the mean ± SD. Differences were calculated with Dunnett's test. Values of $p<0.05$ were considered significant.

RESULTS

Changes in general characteristics

Table I shows the characteristics of experimental rats. DM rats had significantly higher serum glucose ($p<0.001$), and lower body weight (BW) ($p<0.01$) than controls. Absolute heart weight (HW) was low in DM ($p<0.05$).

TABLE I General characteristics of experimental model-rats

Parameter	*Control (n=6) 8th week*	*DM (n=6) 8th week*	*Control (n=6) 12th week*	*DM (n=6) 12th week*
Blood pressure (mmHg)	137±10	142±11	142±6	147±11
Serum glucose (mg/dl)	136±11	554±105[a]	140±10	483±126[a]
Body weight (kg)	0.45±0.03	0.26±0.03[a]	0.60±0.04	0.33±0.04[a]
Heart weight (g)	1.40±0.2	1.00±0.2[b]	1.86±0.21	1.43±0.16[b]
Hcart weight/Body weight (g/kg)	3.15±0.70	3.89±0.89	3.1±0.24	4.3±0.15[c]

Values are means ± SD.

a. $p<0.001$ vs control
b. $p< 0.05$ vs control.
c. $p<0.01$ vs control

TABLE II Echocardiographic findings of experimental model-rats

Parameter	*Control (n=6) 8th week*	*DM (n=6) 8th week*	*Control (n=6) 12th week*	*DM (n=6) 12th week*
IVS /BW(mm/kg)	3.5±0.5	5.5±0.9[a]	2.8±0.1	3.5±0.1[a]
PW/BW (mm/kg)	3.5±0.7	5.6±0.7[a]	3.5±0.1	5.3±0.1[a]
LVDd/BW (mm/kg)	16.4±1.4	26.3±4.1[a]	16.5±2.5	28.9±1.5[a]
LVEF (%)	84.0±3.2	76.4±9.5	91.0±2.4	86.0±3.6
dPWV (mm/sec)	55.5±5.9	37.7±6.5[a]	66.1 ± 12.8	44.5±9.5[a]

Values are means ± SD. (IVS) interventricular septal thickness; (PW) posterior wall thickness; (LVDd) left ventricular diastolic dimension; (LVEF) left ventricular ejection fraction; (dPWV); diastolic posterior wall velocity.

a. $p<0.001$ vs control.

TABLE III Hemodynamic measurements of experimental model-rats

Parameter	*Control (n=6) 8th week*	*DM (n=6) 8th week*	*Control (n=6) 12th week*	*DM (n=6) 12th week*
Heart rate (beats/min)	452±50	404±24[a]	445.0±38.3	374.0±50.8[b]
LVFDP (mmHg)	5.2±2.6	12±6.2[a]	6.1 ±1.9	14.8±4.6[c]
+LV dP/dt (mmHg/sec)	2408±463	1671±382[c]	2017±598	1330±931[c]
-LV dP/dt (mmHg/sec)	2600±981	1914±923	2600±682	1660±598[a]

Values are means ± SD. (LVSP) left ventricular peak systolic pressure; (LVFDP) left ventricular end diastolic pressure.

a. $p< 0.05$ vs control.
b. $p<0.001$ vs control
c. $p<0.01$ vs control

Echocardiographic and hemodynamic findings

Table II shows the echocardiographic findings. Since BW in DM was lower than in controls, the three parameters of LV structure, IVS, PW and left ventricular diastolic dimension (LVD d) were corrected by BW. Both IVS/BW and PW/BW were significantly increased in DM rats compared with controls. A larger LVD d was shown in DM. Although left ventricular ejection fraction (LVEF) rate did not change, decreases in diastolic posterior wall velocity (dPWV) were observed in DM. These results suggest impairment of cardiac function in DM groups. Table III shows the hemodynamic findings. Heart rate (HR) was significantly decreased in DM groups compared with controls ($p<0.05$). DM groups showed elevated left ventricular end diastolic pressure (LVEDP) compared with controls. DM groups had reduced rates of +LV dP/dt. Rates of – LV dP/dt tended to reduce in DM rats after 12 weeks. These results support the data obtained by echocardiograph suggesting cardiac failure in DM groups.

Antioxidant activities

Antioxidant activities were estimated in rat hearts. Figure 1 shows the activity and the expression of Mn-SOD, a key antioxidant enzyme in mitochondria. A decrease in the activity was observed in DM (16.5 ±4.9 milliunits/mg protein, $p<0.05$) at the 8th week compared with the control values at the same week (23.0±3.5 milliunits/mg protein). The activity of Mn-SOD decreased more in DM at the 12th week (12.0±3.7 milliunits/mg protein, $p <0.05$) compared with the control values (23.1±3.0 milliunits/mg protein) (Fig. 1A). On the contrary, the expression of

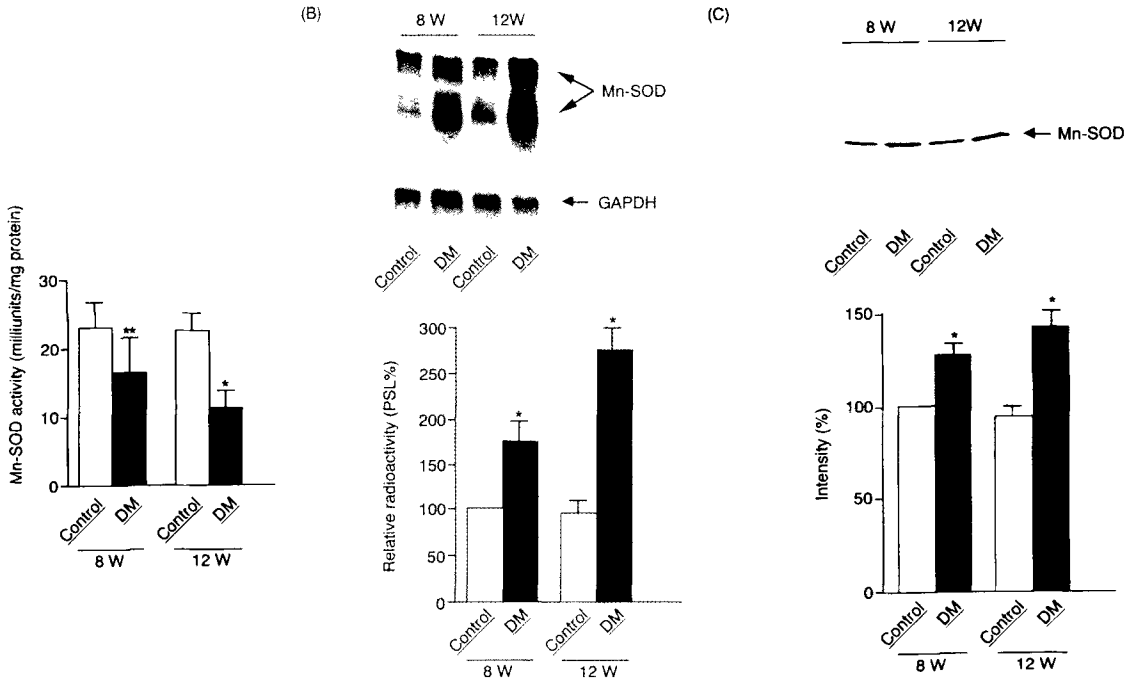


FIGURE 1 The activity and the expression of Mn-SOD. The activity and the expression of Mn-SOD were estimated in the left ventricle of rat hearts. (A), The Mn-SOD activity was measured as described in Materials and Methods. Values are the mean ± SD of six samples. (B), The mRNA levels of Mn-SOD were estimated by Northern blot analysis. The data shown in the diagram were expressed as relative radioactivity (PSL%). (C), Immunological levels of Mn-SOD were estimated by Western-blot analysis. The data shown in the diagram were expressed as intensity (%) when the value at the 8th week was 100%. *Significantly different ($p<0.01$) from the control group. **Significantly different ($p<0.05$) from the control group

Mn-SOD mRNA on Northern blots (Fig. 1B) and Mn-SOD protein on Western blots were different from the activity (Fig. 1C). Levels of Mn-SOD mRNA and its protein were elevated in DM. Figure 2 shows the levels of GSH and the mRNA expression of γ-GCSh. There was a decrease in the levels of GSH in DM at the 12th week (16.2±0.5 nmol/mg protein, $p<0.05$) compared with the control values (21.5±3.0 nmol/mg protein) (Fig. 2A). The expression of γ-GCSh mRNA was concomitantly decreased in DM at the 12th week (Fig. 2B).

Expression of TGF-β1 and type IV collagen

Figure 3 shows the expression of TGF-β1 mRNA. There was a significant increase in the expression of TGF-β1 in DM both at the 8th and 12th weeks. Figure 4 shows the results of immunological staining of type IV collagen in rat hearts at the 12th week. Increase in the expression of type IV collagen was observed around cardiac muscle bundles in DM rats.

DISCUSSION

In the present study, an increase in the wall thickness and in the internal dimensions of LV was observed in DM rat hearts (Tables I–III). These changes were accompanied by cardiac dysfunction including decreases in dPWV and dp/dt, and an increase in LVEDP, which changes are in good agreement with previous

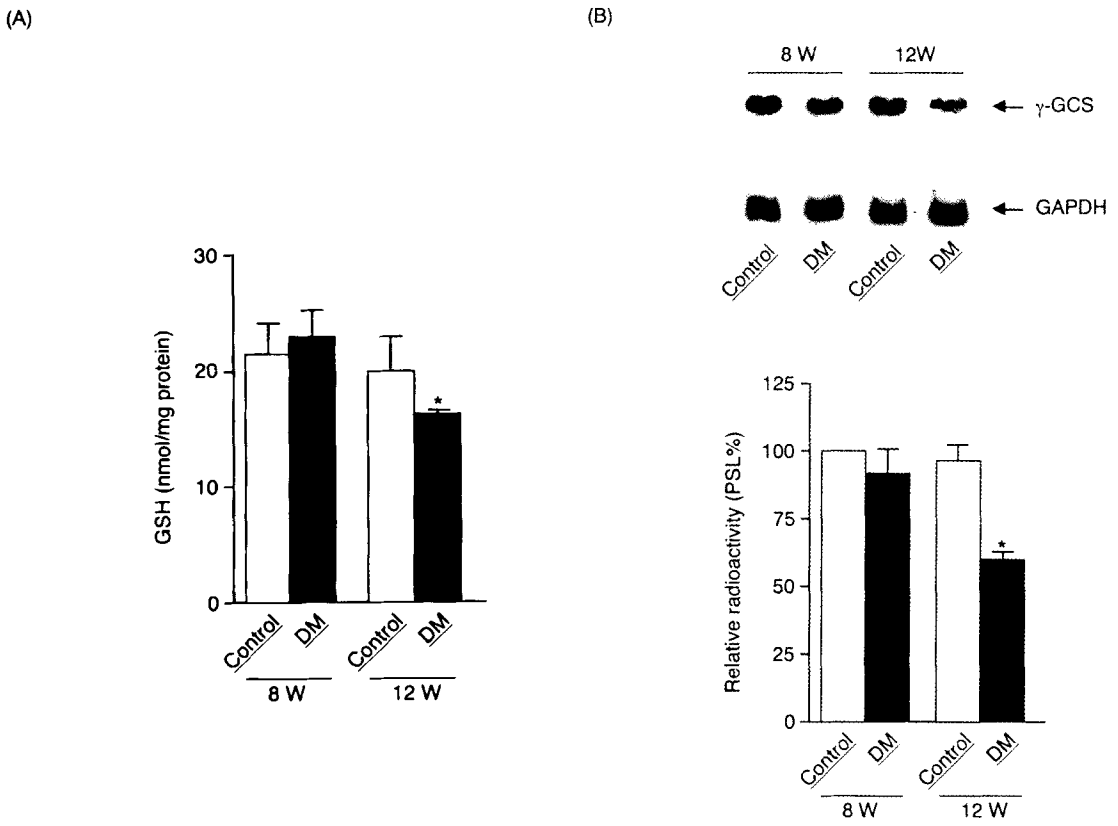


FIGURE 2 The levels of GSH and the expression of γ-GCS. (A), The levels of GSH were estimated in the left ventricle of rat hearts in the 8th and 12th weeks. The homogenized tissue was centrifuged and the supernatant was used as material for the estimation as described in the Materials and Methods. Values are the mean ± SD of six samples (B), The expression of γ -GCSh mRNA was estimated in the left ventricle of rat hearts. The data shown in the diagram were expressed as relative radioactivity (PSL%) when the value at 8th week was 100%. *Significantly different ($p<0.01$) from the control group

report by Litwin *et al.* that LV cavity/wall volume and end-diastolic volume were increased after 26 days in STZ induced-DM rats[5]. As an index of cardiac diastolic dysfunction, we evaluated dPWV, which estimation is believed to be a useful in detecting left ventricular diastolic abnormalities[24]. The abnormalities of cardiac diastolic function in DM rat hearts observed in the present study are in good agreement with a previous report by Penpalgkul *et al.*[25].

Taniguchi *et al.* reported that on glycation of Cu,Zn-SOD resulted in site specific fragmentation and enzymatic inactivation[26]. They further found that glycated Cu,Zn-SOD protein was predominantly observed in DM rat lenses[27]. No change in the activity of Cu,Zn-SOD was observed in DM rat hearts in the present study even at the 12th week (data not shown). The reason of the retain in the Cu,Zn-SOD activity in DM rat hearts is not clear.

Most striking change in DM rat hearts was a decrease in the Mn-SOD activity (Fig. 1). Mn-SOD functions to scavenge oxygen radicals produced in mitochondria and plays a key role in the protection against mitochondrial oxidative stress. Reduction of this activity was thought to reflect the abnormal mitochondrial metabolism of ROS[19]. Myocardial injury was observed in

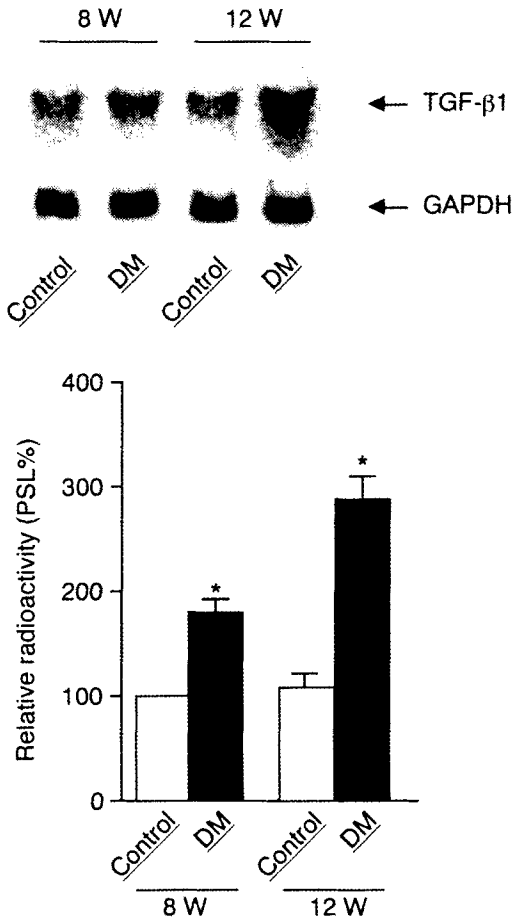


FIGURE 3 Expression of TGF-β1 mRNA. The expression of TGF-β1 mRNA in rat hearts was estimated by Northern blot analysis. The data shown in the diagram were expressed as relative radioactivity (PSL%) when the value at 8th week was 100%. * Significantly different ($p<0.01$) from the control group

Mn-SOD deficient mice[28]. Extensive mitochondrial injury was thought to cause myocyte degeneration leading enlarged and dilated heart. Decrease in the Mn-SOD activity in our study may be a factor for cardiac dysfunction in DM. Mechanisms by which the Mn-SOD activity is reduced are not clear. There are some possibilities. [1], presence of inhibitors; [2], consumption by excess of substrates; [3], modulation of enzyme protein. Our data may suggest presence of inhibitors or excess of ROS in DM mitochondria. Taniguchi *et al.* reported that glycation of Cu,Zn-SOD produced ROS to cause fragmentation of this enzyme protein and enzymatic inactivation[26]. However, change in Mn-SOD molecule was not observed on immunological analysis (Fig. 1C), although inactivation of Mn-SOD by glycation cannot be ruled out.

In contrast, the expression of Mn-SOD protein and mRNA was up-regulated in DM rat hearts (Fig. 1). This is the first report that the expression of Mn-SOD mRNA and protein increased in diabetic condition. Since Mn-SOD mRNA is induced by TNF-α and interleukine-1α (IL-1 α), and oxidative stress[10], such stimuli might present in DM rat hearts.

The expression of γ-GCS is responsive to ROS, cytokines, and hormones. TNF-α and IL-1α stimulate the γ-GCS expression mediated by nuclear factor-κB[16]. Ionizing radiation and oxidized low-density lipoprotein induce the γ-GCS expression mediated by mitogen-activated protein kinases and following activation of activator protein-1 for the cell proliferation[29,30].

The levels of GSH were reduced in DM, especially at the 12th week (Fig. 2). We have reported that hyperglycemic conditions down-regulates the level of GSH and the expression of γ-GCS mRNA[9,16]. Quite recently, down-regulation of GSH synthesis by TGF-β1 was reported in lung epithelial cells[31]. TGF-β1 suppresses the transcription of γ-GCS heavy subunit by unknown mechanism. Impairment of the activities of intracellular signals or transcription factors may be a cause of the decrease. Renal production of TGF-β1 is increased in patients with type II DM[32]. Overexpression of TGF-β1 induces myocardial fibrosis with highly expressed type IV collagen in experimental mice[33]. In DM rats, accumulation of myocardial type IV collagen and alteration in LV diastolic filling were observed[34]. However, no direct evidence for the expression of TGF-β1 has been reported in DM hearts. In the present study, elevation of the expression of TGF-β1 was accompanied with the accumulation of type IV collagen in DM hearts

(A): Control

(B): DM 12W

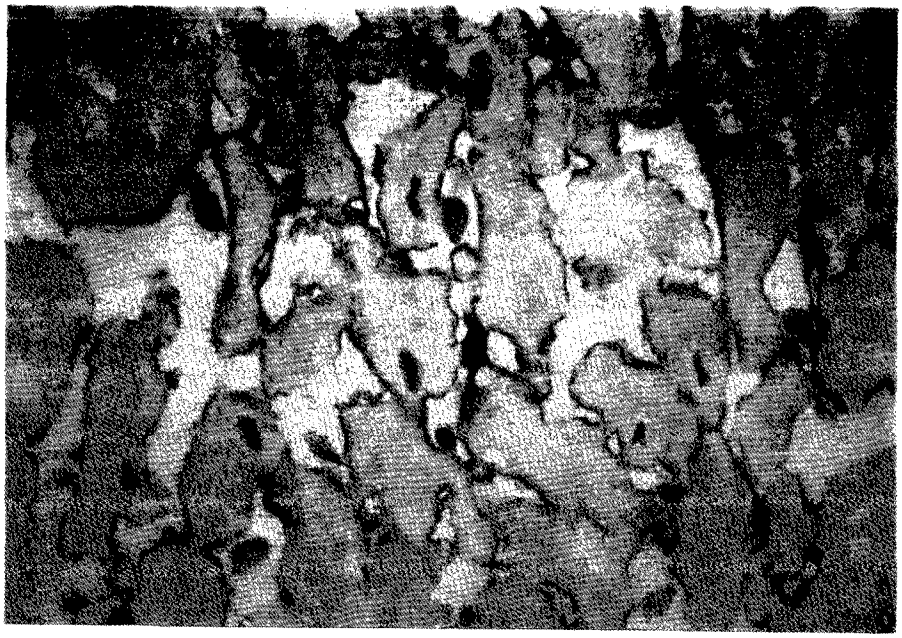
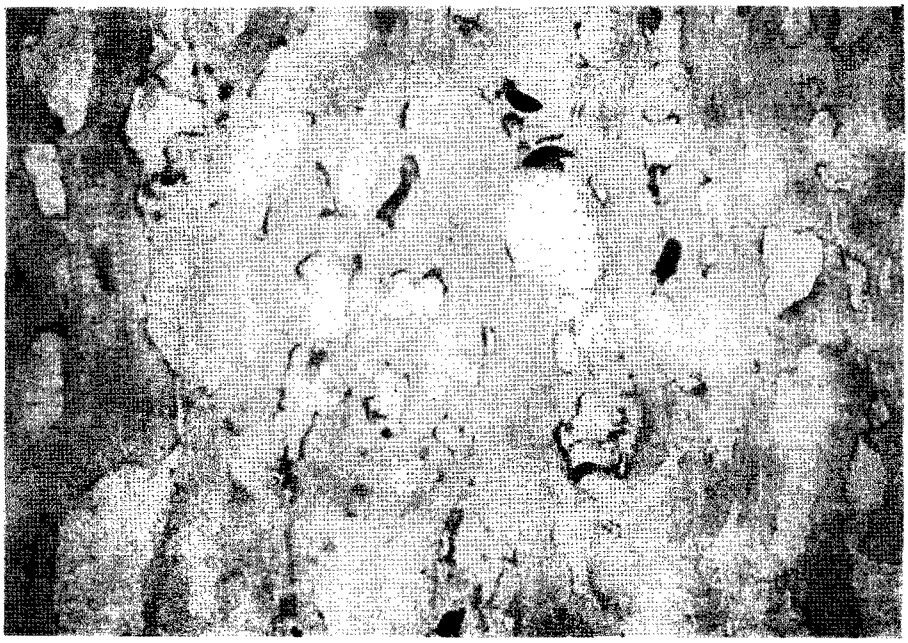
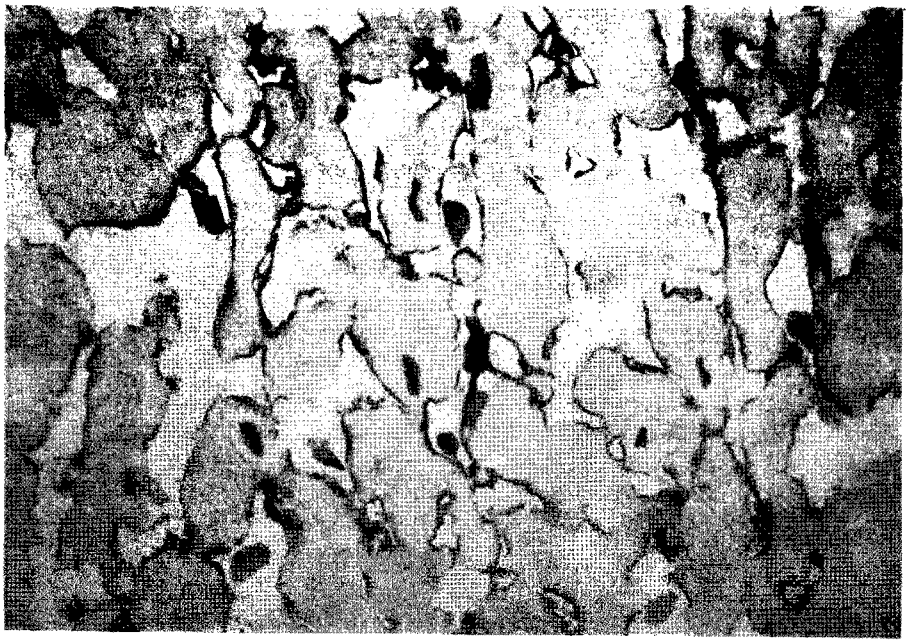


FIGURE 4 Expression of type IV collagen. Type IV collagen was estimated immunohistochemically. Paraffin-embedded samples form rat hearts at 12th week were sectioned and subjected to indirect immunohistochemical staining using anti-type IV collagen antibody and biotin-conjugated second antibody. (A), Control; (B), DM rat at the 12th week. Note accumulation of type IV collagen around cardiac muscle bundles in DM rats (See Color Plate III at the back of this issue)

(A): Control



(B): DM 12W



Color Plate III (See page 259, Figure 4) Expression of type IV collagen. Type IV collagen was estimated immunohistochemically. Paraffin-embedded samples form rat hearts at 12th week were sectioned and subjected to indirect immunohistochemical staining using anti-type IV collagen antibody and biotin-conjugated second antibody. (A), Control; (B), DM rat at the 12th week. Note accumulation of type IV collagen around cardiac muscle bundles in DM rats

(Figs. 3 and 4). This is the first report on the expression of TGF-β1 in DM hearts. It is interesting to speculate that elevation of TGF-β1 causes suppression of the expression of γ-GCS to decrease in the levels of GSH on one hand, and stimulation of the accumulation of type IV collagen to induce fibrous changes, which changes may induce myocardial tissue injury in DM. It has been reported that treatment with probucol, an antioxidant, results in an improvement of the cardiac dysfunction in STZ-induced DM rat hearts[35]. These results suggest that a decrease in the Mn-SOD activity and GSH level reflects irresponsiveness of DM heart to oxidative stress.

In summary, the responsiveness of antioxidants to stress is altered in DM hearts. Especially, decreases in the activity of Mn-SOD and the levels of GSH as well as γ-GCS mRNA were found. Possible role of TGF-β1 was suggested in the suppression of GSH synthesis and accumulation of collagen in DM hearts. Considering that Mn-SOD and GSH are important in cell protection against oxidative stresses, such reduction of antioxidants may relate to the consequent progression of cardiac dysfunction.

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