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Heart and Vessels

A comparison of ultrastructural changes on endomyocardial biopsy specimens obtained from patients with diabetes mellitus with and without hypertension

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Summary. The pathogenesis of diabetic cardiomyopathy is unknown. The synergistic, or enhanced, effect of hypertension on pathological changes in the heart of diabetic patients has been highly suspected. The purpose of this study was to evaluate the myocardial changes related to diabetes mellitus with and without hypertension, using biopsy specimens. We examined the ultrastructural changes in biopsy specimens of the endomyocardium obtained from 25 patients. They were divided into four groups: controls without hypertension or diabetes mellitus (n = 6), and patient with hypertension (n = 3), diabetes mellitus (n = 8), and diabetes with hypertension (n = 8). The diabetic patients showed nearly normal or mildly depressed systolic left ventricular function. Ultrastructural pictures were analyzed for thickening of the capillary basement membrane, presence of toluidine blue-positive materials (i.e., materials showing metachromasia) in the myocytes, size of myocytes, and interstitial fibrosis. The thickening of the capillary basement membrane, the accumulation of toluidine blue-positive materials, and interstitial fibrosis were all significantly greater in the patients with diabetes mellitus compared to the control subjects. The myocytes tended to be small (cell atrophy) in the diabetes group. Although these pathological changes in the heart were characteristic of diabetic patients, irrespective of the presence or absence of hypertension, the presence of hypertension increased the pathological changes of myocardial cells as well as abnormality in the capillary vessels in patients with diabetes mellitus. Alterations in the myocardial cells and capillaries, caused by diabetes mellitus, may lead to myocardial cell injury and interstitial fibrosis and, ultimately, to ventricular systolic and diastolic dysfunction, especially when the diabetes is accompanied by hypertension.

Key words: Biopsy – Diabetes mellitus – Cardiomyopathy – Hypertension

Introduction

A high incidence of diabetes mellitus has been found in patients with congestive heart failure [1–3]. It has been suggested that the presence of diabetes mellitus in the absence of extensive coronary artery disease and hypertension causes left ventricular dysfunction that leads to cardiomyopathy [2, 3]. These observations have given rise to the concept of diabetic cardiomyopathy. The pathogenesis of this cardiomyopathy is unknown, but proposed mechanisms include small vessel (intramural) coronary artery disease [1, 3–5], interstitial myocardial accumulation of glycoprotein and collagen [6], and metabolic alterations of the diabetic myocardium [7, 8].

However, in 1980, Factor et al., who studied postmortem materials as well as animal experiments, claimed that most morphological studies of the diabetic heart have not revealed abnormalities in the myocyte sufficient to account for a reduction in left ventricular function [9]. They suggested that the combined effects of diabetes mellitus and hypertension increase the alterations in myocardial contractility, leading to a specific abnormality of the heart muscle of hypertensive diabetic patients, or to hypertensive-diabetic cardiomyopathy [10–12]. Their studies on hypertensive-diabetic hearts demonstrated severe interstitial fibrosis, focal or confluent scars, and

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extensive myocytolytic activity. However, in 1992, Fischer et al. [13] reported that the primary target of the synergistic damage in hypertensive diabetic heart muscle disease was considered to be the myocardial cell, not the cardiac interstitium. Thus, the pathogenesis of diabetic cardiomyopathy is still a subject of controversy. In addition, ultrastructural changes of the myocytes, interstitium and capillaries, which might occur in diabetics, and modifications due to the presence of hypertension, have not been clarified in previous studies [1–5].

In 1994, an epidemiologic study was carried out by Coughlin et al. [14] to examine the possible role of diabetes mellitus and other factors in the development of idiopathic dilated cardiomyopathy. Their results showed that diabetics are at increased risk for idiopathic dilated cardiomyopathy, particularly in those patients with a history of hypertension. Therefore, it is quite important to clarify morphological changes in the myocytes as well as in the interstitium and coronary microvessels in patients with diabetes mellitus, and to clarify how these changes, if any, differ in the presence or absence of hypertension.

Our object was to study histological changes in the myocardium and capillary bed by ultrastructural methods, using biopsy materials obtained from controls without diabetes mellitus or hypertension, patients with diabetes mellitus or hypertension alone, and patients with diabetes mellitus combined with hypertension, and to investigate the morphological changes due to diabetes mellitus and its modification by hypertension.

Patients and methods

Subjects (see Table 1)

We selected for study 25 subjects, 15 men and 10 women aged 40 to 83 years (mean age: 62 ± 9.3 years) who had undergone diagnostic cardiac catheterization for suspected coronary artery disease because of chest pain and/or discomfort, and/or who had abnormalities on the resting and/or exercise electrocardiogram (ECG). All participants were verified to lack significant coronary stenosis (diameter stenosis ≧75%) on diagnostic coronary angiogram. Criteria for exclusion from the study were history of congestive heart failure, old myocardial infarction, vasospastic angina, congenital and/or valvular heart disease, and the presence of overt systemic disease other than diabetes mellitus and/or essential hypertension. Subjects were classified as hypertensive if the systolic pressure exceeded 150 mmHg and/or the diastolic pressure exceeded 95 mmHg in the supine position, in the morning, at least 3 consecutive days after hospitalization; or if there was a history of documented high blood pressure that required drug therapy prior to hospitalization. Subjects were classified as having diabetes mellitus if the fasting plasma glucose concentration exceeded 120 mg/dl, a 2-h oral glucose tolerance test exceeded 140 mg/dl, or if there was a history of diabetes mellitus that required medical treatment prior to hospitalization. The control group consisted of six subjects with chest pain syndrome and without diabetes or hypertension.

Table 1. Patient characteristics and catheterization data

Parameter	Control	Hypertension	Diabetes	Diabetes with hypertension
n (M/F)	6 (3/3)	3 (2/1)	8 (5/3)	8 (5/3)
Age (years)	59.3 ± 3.6	62.3 ± 9.9	66.0 ± 16.6	60.6 ± 7.4
Diabetes mellitus	−	−	+	+
(years after onset)			(14.1 ± 9.4)	(7.6 ± 6.8)
Hypertension	−	+	−	+
(years after onset)		(15.3 ± 8.2)		(10.8 ± 5.2)
Cardiac catheterization				
LVEF (%)	58 ± 9.3	55 ± 3.5	51 ± 12.7	52 ± 8.7
LVEDVI (ml/m²)	83 ± 19.6	88 ± 8.9	83 ± 25.5	79 ± 9.6
LVP (mmHg)	139 ± 13.9	162 ± 45.8	153 ± 18.8	160 ± 13.3
LVEDP (mmHg)	9 ± 3.9	14 ± 6.2	9 ± 12.4	9 ± 5.3
AOSP (mmHg)	138 ± 10.9	167 ± 28.7	149 ± 23.2	160 ± 10.8
AODP (mmHg)	72 ± 10.2	85 ± 1.0	69 ± 8.8	84 ± 9.6
Mean AOP (mmHg)	95 ± 8.3	113 ± 9.2	98 ± 11.0	113 ± 15.0

LVEF, left ventricular ejection fraction; LVEDVI, left ventricular end-diastolic volume index; LVP, left ventricular peak pressure; LVEDP, left ventricular end-diastolic pressure; AOSP, systolic aortic pressure; AODP, diastolic aortic pressure; Mean AOP, mean aortic pressure

Histological examination

Endomyocardial biopsy was performed from at least three sites of the right ventricular septum, using biopsy forceps 501–300 (Cordis, Miami, FL, USA). Part of the biopsy specimen was fixed in 10% formalin and processed routinely. Paraffin sections (3 μm thick) were stained with hematoxylin–eosin and Azan stain (Mallory-Heidenhain) for connective tissue. Some of the biopsy materials were fixed with 2% glutaraldehyde and postfixed with 1% osmium tetroxide. The tissues were transferred through a graded alcohol series followed by propylene oxide, and embedded in Epon 812. Semi-thin epon sections were stained with toluidine blue, Alcian blue or periodic acid-Schiff (PAS) for the detection of mucopolysaccharides. Ultrathin epon sections were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (JEOL-1200EX, Japan).

Light and electron microscopic analysis was performed quantitatively or semi-quantitatively, based on the histological sections obtained from each subject. The main items for examination were as follows. (1) Thickness of the capillary basement membrane: 20 capillaries with a spherical shape in each case (one specimen) in electronmicroscopic pictures were measured for this purpose and the values in each specimen were then averaged according to the method of Siperstein et al. [15]. (2) Deposits of toluidine blue-positive materials (i.e., materials showing metachromasia) in the cytoplasm of the myocytes were graded from 0 to 5+ in 10 serial semithin sections in each case, based on their frequency and/or magnitude in a 2 mm-square block, i.e., 0, no toluidine blue-positive materials; 1+–2+, minimally increased; 3+, moderate changes; 4+–5+, severe changes. (3) The extent and degree of fibrosis, evaluated in 10 serial semithin sections in each case, from a block that was also 2-mm square, was subjectively graded on the following scale [16, 17]: grade 0 signified no apparent collagen fiber proliferation except for small islets of fibrous tissue around the capillaries and an intercellular single layer of collagenous tissue as in normal myocardium. Focal and minimal fibrosis was graded (1+) and the most prominent fibrosis, covering more than half the area of the specimen, was classified as (4+). Grades (2+) and (3+) are intermediate between (1+) and (4+). (4) The size of myocytes: the shortest diameter of the myocytes was measured only in nucleated transverse sections stained with toluidine blue. Thirty myocytes in each biopsy specimen were measured with an ocular micrometer disc with a linear scale, and the average myocyte diameter of each specimen was calculated. According to two reports [17, 18], the normal average myocyte diameter ranges from 10 to 16 μm. Average myocyte diameters of 16.01 to 20 μm are graded (1+), those of 20.01 to 24 μm (2+), those of 24.01 to 28 μm (3+), and those over 28 μm (4+).

The patients' biopsy materials were divided into four groups: diabetes mellitus group (n = 8), hypertension group (n = 3), diabetes mellitus accompanied by hypertension (n = 8), and a control group with chest pain syndrome but without diabetes and hypertension (n = 6). Tissue pathology in each group was compared with respect to the criteria described above. Written informed consent was obtained from all patients before the study.

Data analysis

Values were expressed as mean ± SD. Statistical comparisons of the four groups were performed using one-way analysis of variance followed by the post-hoc test (Fisher's Protected Least Significant Difference). Differences were considered statistically significant if $P < 0.05$.

Results

Although left ventricular ejection fraction tended to be lower in the diabetes or the diabetes with hypertension group, there were no significant differences in hemodynamic variables, including ejection fraction obtained from the cardiac catheterization study, among the four groups (Table 1).

Typical electron micrographs of the capillary basement membrane are shown for the control (Fig. 1a), diabetic (Fig. 1b) and diabetic plus hypertension subjects (Fig. 2). The mean thickness of the capillary basement membrane was 75 ± 15 nm in the control group, 67 ± 8 nm in the hypertension group, 118 ± 40 nm in the diabetes group and 153 ± 48 nm in the diabetes with hypertension group. The thickness in the diabetes and the diabetes with hypertension groups was of a significantly higher grade than in the hypertension and the control groups. However, there was no significant difference between the diabetes and the diabetes with hypertension groups (Table 2). Pericytes were observed in most cases.

Toluidine blue-positive materials showing metachromasia were observed in the cytoplasm of the myocytes in most cases of diabetes (Fig. 3a). The grade indicating the frequency of the appearance of toluidine-blue positive materials was 2.0 ± 0.5 in the control group, 1.6 ± 0.4 in the hypertension group, 3.4 ± 0.9 in the diabetes group, and 3.9 ± 1.1 in the diabetes with hypertension group. The grade in the diabetes and the diabetes with hypertension groups was significantly higher than that of the hypertension and control groups

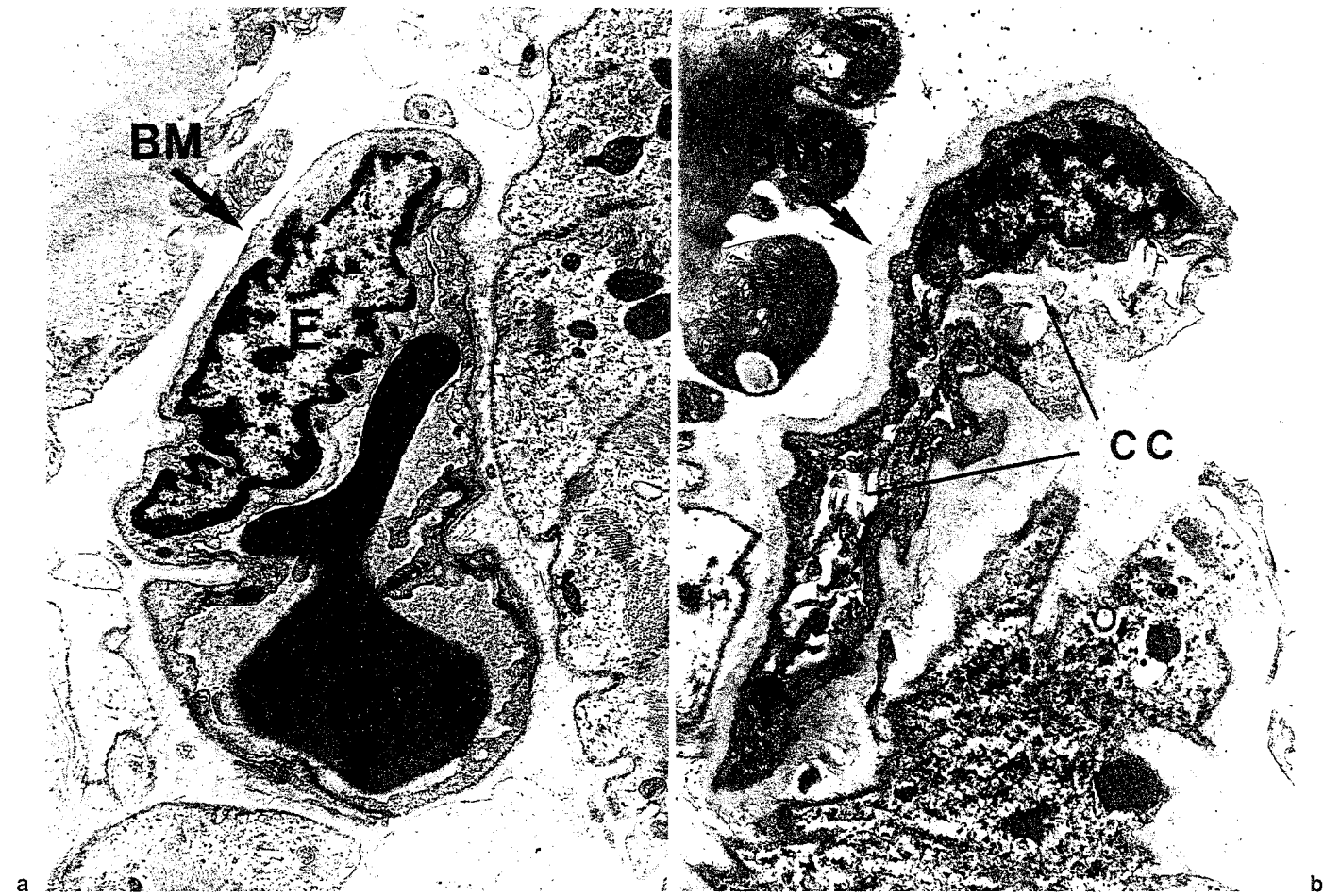

Fig. 1a,b. Micrographs. **a** Micrograph of a capillary in the control subject showing a normal basement membrane (×13400). **b** Micrograph of a capillary in the diabetes mellitus patient showing a thick basement membrane (×13700). *BM*, basement membrane (*arrows*); *CC*, capillary cavity; *E*, endothelial cell

($P < 0.05$, Table 2). There was no significant difference in this respect between the diabetes and the diabetes with hypertension groups. Positive staining with Alcian blue and PAS was observed at the periphery of the cytoplasm in a similar distribution to that of the toluidine blue-positive materials mentioned above (Fig. 3b). Electron microscopic examination of the toluidine blue-positive materials revealed mixtures of homogeneous gelatinous substances and granular substances with a high electron density (Fig. 3c). The fibrous tissue frequently surrounded individual or groups of muscle cells, isolating them. We observed that the interstitium was stained blue by Azan stain. Fibrotic lesions were more common in the hypertensive-diabetic and diabetic hearts (Fig. 4) in comparison with the control and hypertensive groups. Of the 8 diabetic patients, 3 had 1+, 2 had 2+, and 1 had 3+ interstitial fibrosis (Table 2), and 4 patients in the diabetes with hypertension group had 1+, 2 had 2+, and 1 had 3+ interstitial fibrosis. The mean diameter of the myocytes was 12.2 ± 0.5 μm in the control group, 13.7 ± 0.8 μm in the hypertension group, 9.0 ± 1.7 μm in the diabetes group, and 11.9 ± 2.0 in the diabetes with hypertension group. There was no significant difference in the diameter size among the groups, but it tended to be small in the diabetes group, compared with the control group.

Discussion

We investigated the ultrastructural changes in patients with diabetes mellitus with nearly normal or mildly depressed systolic left ventricular function. Our main findings were that the thickness of the capillary basement membrane, the incidence of toluidine blue-positive materials showing metachromasia in the myocyte, and interstitial fibrosis were significantly

greater in the patients with diabetes mellitus than in the nondiabetic subjects. In previous studies [9–11, 14], ultrastructural analysis of the myocytes and interstitial tissue, as well as of the microcirculation, was performed in diabetic patients using autopsy and biopsy specimens, but no significant abnormalities in the myocytes which would account for myocardial disease were revealed. The ultrastructural changes as well as other pathological changes found in the present study may play a role in inducing cardiac dysfunction or lead to a background for the induction of diabetic cardiomyopathy throughout the course of diabetes mellitus.

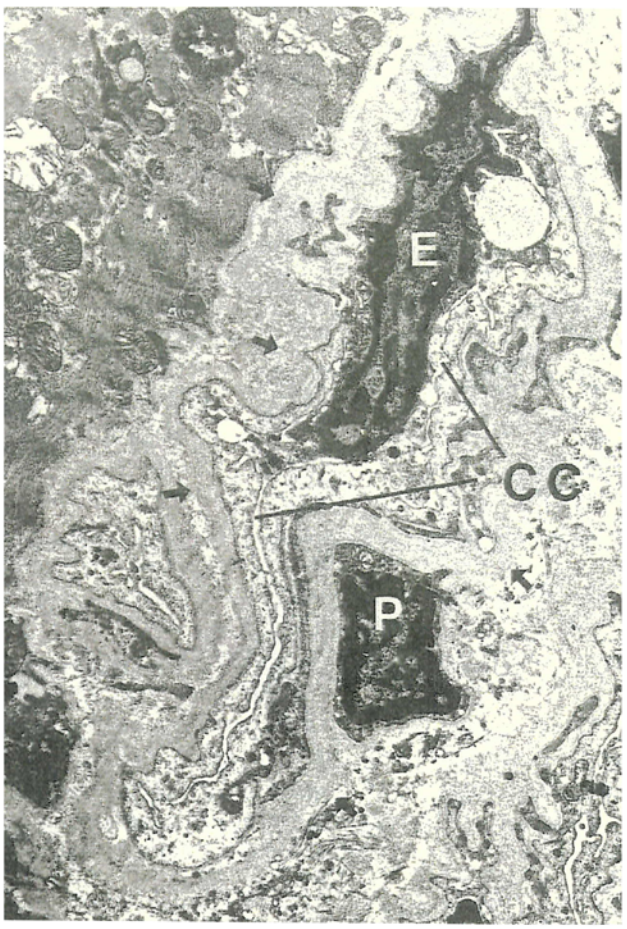

Fig. 2. Micrograph of capillaries from a hypertensive diabetic patient. Capillaries with a thick basement membrane (*arrows*) show severe stenosis. *CC*, capillary cavity; *P*, pericyte; *E*, endothelial cell (×12600)

Thickening of the capillary basement membrane is consistently found in the skin [19], kidney [20], retina [21], as well as other tissues [22] of patients with diabetes mellitus. Fischer et al. also demonstrated thickened capillary basement membrane in biopsy samples of myocardial tissue obtained during coronary artery bypass surgery in patients with diabetes mellitus [23]. The average width of the capillary basement membrane in that study resembled that observed in the patients with diabetes mellitus in the present study. Further, we found the thickness of the capillary basement membrane to be significantly greater in the hypertensive diabetics and the diabetics without hypertension than in the nondiabetic subjects. This result suggests that hypertension as well as diabetes mellitus may also be an independent factor affecting transcapillary dynamics or fluid transport and/or myocardial metabolism.

There are a few reports describing the accumulation of toluidine blue-positive materials showing metachromasia mainly in the cytoplasm of the myocytes in diabetic patients. We have excluded the possibility of an artifact, as our biopsy specimens were processed rapidly after extirpation, and by a similar method in all cases. The toluidine blue-positive materials showing metachromasia consisted of homogeneous gelatinous substances and granular substances with a high electron density. As they were also stained by Alcian blue and PAS, we suspected they consisted of acid mucopolysaccharide. PAS-positive materials have previously been demonstrated in the walls of small

Table 2. Histological findings

Parameter	Control ($n = 6$)	Hypertension ($n = 3$)	Diabetes ($n = 8$)	Diabetes with hypertension ($n = 8$)
BM thickening (nm)	75 ± 15	67 ± 8	118 ± 40*	153 ± 48*,**
Toluidine blue-positive materials	2.0 ± 0.5	1.6 ± 0.4	3.4 ± 0.9*	3.9 ± 1.1*
Diameter of myocytes (μm)	12.2 ± 0.5	13.7 ± 0.8	9.0 ± 1.7	11.9 ± 2.0
Interstitial fibrosis	0.33 ± 0.38	0.33 ± 0.58	1.25 ± 0.73*	1.37 ± 0.70*

BM, capillary basement membrane
* $P < 0.05$ vs control and hypertension; ** $P < 0.05$ vs diabetes mellitus

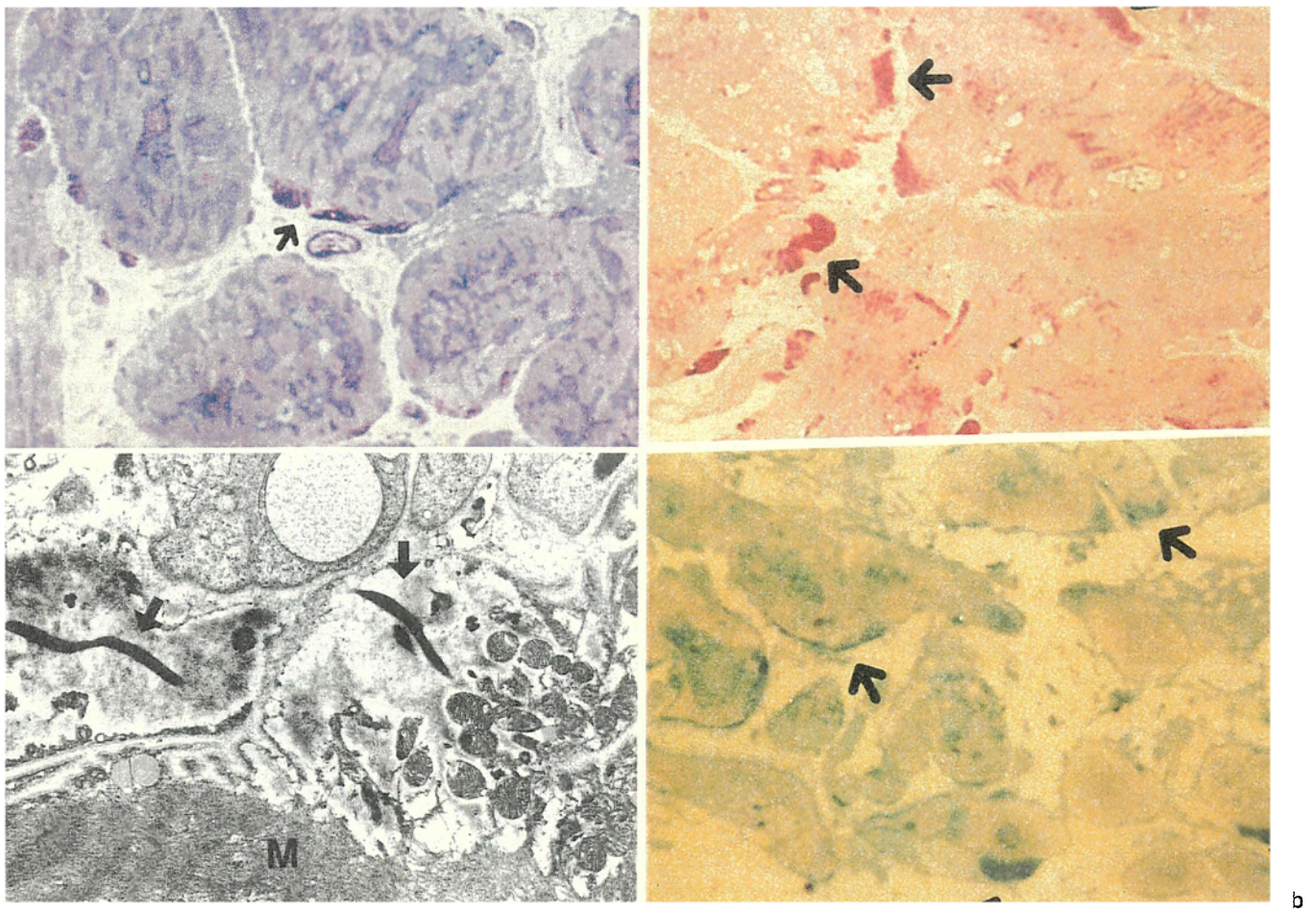

Fig. 3a–c. Micrographs. **a** Micrograph depicting toluidine blue staining of cardiac muscle tissue from a patient with diabetes mellitus. *Arrows* indicate abnormal areas with toluidine blue staining. *M*, myocardial cell (×40). **b** The upper plate shows periodic acid-Schiff (PAS) staining of cardiac muscle tissue in a patient with diabetes mellitus. *Arrows* indicate abnormal areas with PAS staining. The lower plate shows Alcian blue staining of cardiac muscle tissue in a patient with diabetes mellitus. *Arrows* indicate abnormal areas with Alcian blue staining (×40). **c** Micrograph showing ultrastructural findings stained with toluidine blue. The lesions are composed of mixtures of homogeneous gelatinous substances and granular/linear substances with a high electron density (*arrows*). *M*, myocardial cell (×14000)

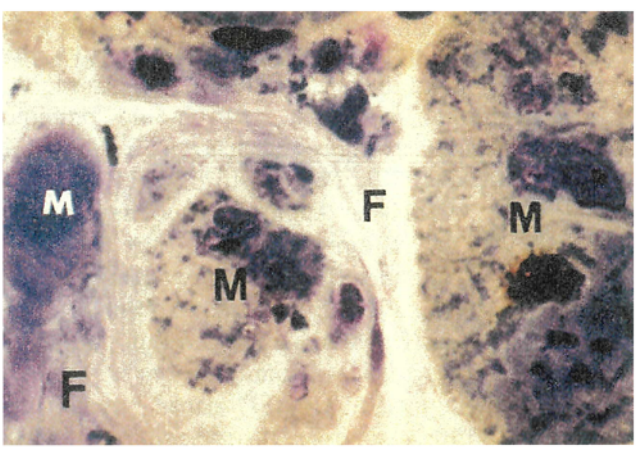

Fig. 4. Micrograph of interstitial fibrosis from cardiac muscle tissue in a patient with diabetes mellitus. *M*, myocardial cells; *F*, areas of interstitial fibrosis (×100)

coronary arteries and in the perivascular interstitial tissues, and have been linked to the genesis of cardiac microangiopathy in patients with diabetes mellitus [1, 2]. It is unclear whether the toluidine blue-positive substances showing metachromasia observed in the present study are identical to the PAS-positive substances previously shown in the arterioles, as we did not observe the small coronary artery, but only the endomyocardial capillary. It is probable that the toluidine blue-positive material showing metachromasia could also consist of metabolites resulting from abnormal metabolic pathways in the myocytes in diabetes mellitus. Metabolites associated with hyperglycemia, such as the sorbitol produced by the activation of the polyol metabolic pathway, accumulate in the nerve and retina [24, 25]. This could account for the finding of PAS-reactive deposits in the hearts of

patients with diabetes mellitus. While we did not observe any closed capillaries in our specimens, we did find a high incidence of luminal stenosis with a thick capillary basement membrane in the diabetic patients. We speculate that such capillary deformities would increase vascular resistance and impede blood flow, and the thickening of the capillary basement membrane would further disturb myocyte perfusion and impair myocyte function, leading to myocardial injury. However, it should be noted that capillary internal area was measured using biopsy materials fixed at the isobaric state. According to previous results [26, 27] obtained in the in situ coronary circulation, the pressure difference between small coronary arterioles and coronary veins was approximately 50 mmHg, although intracapillary pressure has not been reported so far. It is unclear how capillary stenosis was exacerbated in the absence of intraluminal pressure, but we must take into account this factor when applying the present results to clinical settings.

Hypertension is considered to be an important cofactor in the development of diabetic cardiomyopathy, as suggested from our results and those of previous studies in which diabetic patients who died of congestive heart failure also had hypertension [9]. The ventricular wall of the mammalian heart can achieve a striking enlargement, principally through hypertrophy of myocytes and hyperplasia of interstitial fibroblastic cells, when an increased work load is imposed for a long time by hypertension [18]. In this study, however, the myocytes tended to be small in the diabetes group and were within the normal range in the diabetes with hypertension group, suggesting that myocytes in diabetes might become atrophic, probably due to metabolic disturbance. Clinical and experimental studies [10, 28] have shown that interstitial and replacement fibrosis are more marked in the hearts of hypertensive diabetics than in patients with only diabetes or hypertension. Further, normotensive diabetics showed only minimal pathological changes in interstitial fibrosis. Thus, these authors suggested that the combination of hypertension and diabetes mellitus is likely to lead to cardiac dysfunction followed by cardiac failure. However, we observed interstitial fibrosis to be greater not only in hypertensive-diabetic but also in diabetic hearts compared to hypertension and control hearts. That is, our present findings indicated that specific and definite pathological changes occur in the heart with diabetes mellitus, even in the absence of hypertension. We also found the accumulation of abnormal materials showing metachromasia in the myocytes of diabetic patients as well as in hypertensive diabetics, in which a normal diameter was observed despite the presence of hypertension, as mentioned above. Although Fischer et al. [13] reported that the primary target of the synergistic damage in hypertensive diabetic heart muscle disease is the myocardial cell, not the cardiac interstitium, the results of the present study indicate that not only myocardial cell abnormality but also proliferation of the cardiac interstitium is important in the induction of diabetic cardiomyopathy. Moreover, it should be noted that the long-standing diabetic state itself introduces pathological changes in the myocardium, and the presence of hypertension enhances these abnormalities.

In conclusion, in cardiac biopsy specimens taken from diabetics, histological changes found in the myocytes, such as the deposit of toluidine blue-positive materials showing metachromasia and a tendency for the myocytes to be small, suggesting cell atrophy, were accompanied by interstitial fibrosis and structural changes of the capillary vessels. The role of the interaction between the myocytes, interstitium, and capillaries regarding the individual morphological changes is not clear, but each interaction is likely to occur and lead to a further deterioration of the individual pathological changes, especially when hypertension is present. These combined histological changes in the heart would then lead to systolic and diastolic ventricular dysfunction. However, such a hypothesis was not verified in the present study, and further investigation is required to prove this possibility.

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