Changes in Extracellular Matrix Components in Cardiomyopathic Syrian Hamster, BIO 14.6

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We examined changes in the distribution of extracellular matrix components in the myocardium of cardiomyopathic Syrian hamsters (BIO 14.6). Fibronectin, laminin, and type IV collagen, and type I and III collagens were immunohistochemically stained by the avidin-biotin-peroxidase complex method, using a polyclonal antibody for each component. Hearts obtained from 4 stages of BIO 14.6 cardiomyopathy were examined.

Peri- and endomysial fibrosis increased as the disease progressed. Replacement and meshwork (perimysial fibrosis penetrating the intercellular space) fibrotic lesions appeared beginning in the 2nd stage, ie, the fibrotic and healing stage.

All of the components examined, ie, fibronectin, laminin and type IV collagen, and type I and III collagens, were present in various fibrotic lesions and played a significant role in fibrotic changes throughout all of the stages of the disease. No primary deficit of any of these components was seen.

An increased distribution of fibronectin was observed in both the enlarged peri- and endomysial spaces beginning in the initial stage, ie, the necrotic stage, when myocyte hypertrophy was inconspicuous, and distribution throughout the myocardium increased further as the disease progressed.

Laminin and type IV collagen in the fibrotic lesions were not restricted to the myocyte membrane.

Type III collagen was distributed in replacement and meshwork fibrotic lesions, and the extent of its distribution increased in proportion to that of type I collagen.

The continuous increases in the distribution of fibronectin, laminin and type III collagen indicate that fibrotic changes occurred continuously in this model. The deposition of type I and III collagens and fibronectin before myocyte hypertrophy suggests that the fibrotic changes in this model were not related to myocyte hypertrophy in the early stage. (*Jpn Circ J* 1995; **59**: 631–640)

IDIOPATHIC dilated cardiomyopathy (DCM) is a disorder of unknown etiology, characterized by left ventricular (LV) cavity enlargement and LV wall thinning, in

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association with reduced function. The major histopathological characteristics of DCM are myocyte degeneration and cardiac fibrosis^{1,2}

Since several biological studies have revealed the biological activity of various extracellular matrix components, including glycoproteins such as fibronectin^{3,4} laminin^{5–7} and several types of collagens, a considerable amount of attention has been

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focused on the role played by these components in the pathology of heart disease. Indeed, it has been suggested that a primary deficit of extracellular matrix components is implicated in hereditary heart diseases⁸

The cardiomyopathic Syrian hamster⁹ is a reproducible, well-characterized model of cardiomyopathy that shows 4 distinct pathological phases in the development of the disease: 1) myocyte necrotic stage (30–100 days), 2) fibrotic and healing stage (100–160 days), 3) hypertrophy, dilation, and scar stage (160–200 days), and 4) continuing dilation and terminal heart failure stage (200–300 days). Therefore, this model is useful for examining extracellular matrix abnormalities in various pathological conditions.

Although a recent study has indicated connective tissue abnormalities in cardiomyopathic hearts,¹⁰ to our knowledge, there have been no studies in which the distribution of extracellular matrix components was examined immunohistochemically in such hearts. In this study, we used immunohistochemical staining to investigate the localization of the extracellular matrix components fibronectin, laminin, and type IV collagen (the latter two are basement membrane components), and two major extracellular collagen components, ie, type I and III collagens.

MATERIAL AND METHODS

Animals

The experimental animals were male Syrian hamsters, strain BIO 14.6. We examined 3 animals in each stage of cardiomyopathy, (necrotic stage, 63–70 days; fibrotic and healing stage, 147–161 days; hypertrophy, dilatation, and scar stage, 224–231 days; and continuing dilation and terminal heart failure stage; 308–343 days). A corresponding strain of non-diseased hamster, BIO F1b, served as the control, and 3–4 animals were used at each stage.

Tissue Preparations

The total body weight of each hamster was measured, and the animals were sacrificed. The heart was removed and the adhesions were carefully dissected away. The heart was then weighed and cut horizontally at the level of the papillary muscle. The basal half

was used for conventional light microscopic examination and the apical half was used for immunohistochemical staining.

Conventional Light Microscopic Examination

The excised heart was fixed with 10% buffered formalin and embedded in paraffin. The paraffin-embedded specimen was then cut into 6 μ m-thick sections, which were stained with hematoxylin-eosin and Azan-Mallory.

Immunohistochemistry

For the immunohistochemical study, we used the avidin-biotin-peroxidase complex (ABC) method.¹ The excised hearts were immediately frozen in acetone, which was cooled with dry ice. The frozen hearts were cut into 6 μ m-thick sections using a cryostat. These cryotome sections were then air-dried for 30 min and immersed in methanol containing 0.3% hydrogen peroxide for 30 min to completely block endogenous peroxidase activity. To avoid non-specific reaction to the primary antibody, the sections were treated with 1.5% normal goat serum for 30 min at room temperature. After 3 washes with phosphate-buffered saline, the sections were incubated with the diluted primary antibody for an appropriate amount of time at 4°C. The primary antibodies used in this study were: rabbit anti-bovine-fibronectin polyclonal antibody (LSL, Tokyo, Japan), rabbit anti-mouse laminin polyclonal antibody (LSL, Tokyo, Japan), rabbit anti-bovine type IV collagen polyclonal antibody (Chemicon, California, USA), rabbit anti-human type III collagen polyclonal antibody (Chemicon, California, USA), and rabbit anti-bovine type I collagen polyclonal antibody (Advance, Tokyo, Japan). For incubation with the sections, the primary antibodies against fibronectin, laminin and type IV collagen, and type I and III collagens were diluted 1:500 (2 μ l/ml), 1:1000 (1 μ l/ml), 1:500, 1:40 (25 μ l/ml), and 1:500, respectively, in phosphate-buffered saline containing 1.5% normal goat serum. Incubation with the primary antibody was performed at 4°C for 20 h.

After incubation with the primary antibody, the section was treated for 2 h at room temperature with the second antibody: anti-rabbit IgG serum diluted 1:2000 in

TABLE I HEART WEIGHT, TOTAL BODY WEIGHT, AND HEART/BODY WEIGHT RATIO

	Stage			
	Necrotic	Fibrotic and Healing	Hypertrophy, Dilation and Scar	Dilation and Terminal Heart Failure
<i>Control Hamster</i>				
Heart Weight (mg)	380±66	459±65	550±28	652±134
Total Body Weight (g)	105±22	128±10	144±8	170±10
Heart/Body Weight Ratio	3.7±0.6	3.6±0.5	3.8±0.6	3.8±0.6
<i>Cardiomyopathic Hamster</i>				
Heart Weight (mg)	324±38	502±82	723±139	808±257
Total Body Weight (g)	70±5	111±12	90±21	119±11
Heart/Body Weight Ratio	4.6±0.5	4.6±0.9	8.6±2.8	6.8±2.0

Values are mean ± SD

TABLE II IMMUNOHISTOCHEMICAL STAINING

		Stage			
		Necrotic (Fig. 1A–E)	Fibrotic and Healing	Hypertrophy, Dilation and Scar	Dilation and Terminal Heart Failure (Fig. 2A–E)
Endomysium	FN	+	+	++	++
	LM	++	++	++	++
	IV	+	+	+	++
	III	++	++	++	++
	I	+	+	+	+
Perimysium	FN	++	++	++	++
	LM	—	—	+	+
	IV	—	—	+	+
	III	++	++	++	++
	I	++	++	++	++
Replacement Fibrotic Lesion	FN	/	++	++	+++
	LM	/	+	+	++
	IV	/	+	+	++
	III	/	++	++	+++
	I	/	++	++	+++
Meshwork Fibrotic Lesion	FN	/	+	++	+++
	LM	/	—	+	++
	IV	/	—	+	++
	III	/	++	++	+++
	I	/	++	++	+++

FN; fibronectin, LM, laminin, IV, type IV collagen, III; type III collagen, I; type I collagen.

phosphate-buffered saline containing 1.5% normal goat serum.

The sections were then reacted with ABC, and finally stained with diaminobenzidine.

To stain the cell nucleus, the sections were incubated with 0.1 mol/L veronal acetate-buffered 1% methyl green for 10 min.

Immunohistochemical Control Procedure

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The following procedures were performed: 1) negative control staining with normal rabbit serum instead of each primary rabbit antibody was performed for each staining; 2) immunoabsorption test using each purified component corresponding to the antibody was performed in sections obtained from hearts in the continuing dilation and terminal heart failure stage; and 3) to examine the

Fig.1. Immunohistochemical findings during the necrotic stage in cardiomyopathic hearts. A; fibronectin ($\times 150$), B; laminin ($\times 150$), C; type IV collagen ($\times 150$), D; type III collagen ($\times 150$), E; type I collagen ($\times 150$), F; negative control staining ($\times 150$).

No positive staining is observed in negative control staining (F). In contrast, positive staining (brown color) is observed to various degrees in A-E.

specificity of antibodies, skin obtained from control hamsters was stained with fibronectin, laminin and type IV, I and III collagens, liver was stained with fibronectin and type I and III collagens, and kidney was stained with laminin and type IV collagen.

Analysis

Since the extracellular matrix components of the heart are located in the epi-, peri-, and endomysium¹²⁻¹⁴ and in lesions where fibrosis occurs to replace myocyte necrosis (replacement fibrotic lesions), we analyzed

Fig.2. Immunohistochemical staining of cardiomyopathic hearts in the continuing dilation and terminal heart failure stage. A; fibronectin ($\times 150$), B; laminin ($\times 150$), C; type IV collagen ($\times 150$), D; type III collagen ($\times 150$), E; type I collagen ($\times 150$). All of the components examined were deposited with increased distribution. Arrows (E) indicate meshwork fibrotic lesions.

the localization of each of the extracellular matrix components in these regions. When perimysial fibrosis progressed, it penetrated irregularly into the intercellular space, resembling meshwork. This fibrotic lesion, which we defined as a meshwork fibrotic lesion (a typical meshwork lesion is

indicated by the arrow in Fig. 2E), was also analyzed.

The extent of staining was graded as follows; —, no staining; +, slight but apparent (Fig. 1C); ++, moderate (Fig. 1B); +++, strong (Fig. 2E). Initially, a reference photograph was made for each grade of

staining. Inter- and intraobserver differences were checked using 50 sample photographs. For each section, 5 fields ($\times 200$ magnification) were analyzed. All assessments were performed by 2 investigators, one blinded to the identity of the samples, and the other an experimenter. When the two investigators disagreed, an additional blinded observer checked the samples.

RESULTS

Heart/Body Weight Ratio

The heart/body weight ratio was higher in the cardiomyopathic Syrian hamsters than in control hamsters, as shown in Table I. This ratio increased with age in the cardiomyopathic Syrian hamsters, although it varied substantially between animals.

Immunohistochemical Findings

Control Hamsters: In the control hamsters, BIO F1b, fibronectin was found homogeneously in the peri- and endomysial spaces. Laminin and type IV collagen surrounded myocytes in a linear ring pattern. Type I and III collagens were observed in a fibrillar pattern in both the peri- and endomysial spaces. The fibrous staining pattern of type I collagen in the endomysial spaces was thinner than that of type III. The distribution of staining for these components remained essentially unchanged throughout the course of the observations.

The distribution of fibronectin, laminin, type IV, I and III collagens in the skin was the same as that previously reported¹⁵. Similarly, in the liver (fibronectin and type I and III collagen were stained) and kidneys (laminin and type IV collagen were stained), staining patterns similar to those reported previously¹⁶⁻¹⁹ were observed.

Syrian Cardiomyopathic Hamsters (BIO 14.6): Table II summarizes the results of immunohistochemical staining.

Necrotic Stage (30–37 days); Fig. 1A–E

Light microscopic examination showed vacuolization and hypertrophy in several myocytes. Scant focal necrosis was observed. Neither peri- nor endomysial spaces were widened.

Immunoactivity for fibronectin was present in both peri- and endomysial spaces.

Laminin and type IV collagen surrounded myocytes, ie, these constituents were present in endomysial spaces. Both type I and III collagens were observed in the peri- and endomysial spaces. These staining patterns did not differ from those in control hamster hearts, except that the fibrillar staining pattern of type I collagen in the perimysial space was irregular and coarse compared with that in control hearts.

Fibrotic and Healing Stage (144–147 days)

Light microscopic examination revealed increases in the perimysial spaces associated with fibrosis. Myocyte disarray was detected and the endomysial space was also enlarged. Azan-Mallory staining showed fibrotic changes in these enlarged peri- and endomysial spaces. Meshwork fibrotic lesions appeared. Focal fibrotic lesions where myocyte necrosis had occurred, ie, replacement fibrotic lesions, were also observed.

Immunoactivity for fibronectin was found in both meshwork and replacement fibrotic lesions. Its staining pattern was not fibrous and it was deposited non-homogeneously.

Laminin and type IV collagen appeared in enlarged intercellular spaces and some replacement fibrotic lesions, but not in the perimysial space. These components were observed in replacement fibrotic lesions even where staining was not restricted to myocyte membranes.

Both type I and III collagen immunoactivity was observed in meshwork fibrotic lesions in a fibrous staining pattern. Immunoactivity for type I and III collagens was also present in the replacement fibrotic lesions.

Hypertrophy, Dilation, and Scar Stage (224–231 days)

Peri- and endomysial spaces were more enlarged than in earlier stages. Light microscopic examination revealed apparent myocyte hypertrophy.

Immunoactivity for fibronectin was observed in the meshwork fibrotic lesions. The extent of the distribution of fibronectin had also increased in these lesions.

Laminin, associated with type IV collagen, appeared in the meshwork fibrotic lesions, and neither was limited to the myocyte membrane.

Both type I and III collagens were

observed in the meshwork fibrotic lesions. Their fibrous staining pattern and distribution had increased in thickness.

Continuing Dilation and Terminal Heart failure Stage (308–347 days); Fig. 2A–E

Conventional microscopic examination showed progression of myocyte degeneration and a diffuse increase in fibrosis. The thickness of the left ventricular wall was considerably reduced.

The localization of fibronectin was similar to that in the previous stage, but the extent of its distribution had increased. The staining pattern of laminin, as well as that of type IV collagen, in the replacement fibrotic lesions became wavy and the distribution increased.

The localization of type I and III collagens was similar to that in the previous stage, but the distribution had increased.

The staining pattern of fibronectin, laminin, and type IV collagen, and that of type III and I collagens, was thicker around hypertrophic myocytes than around relatively less hypertrophic myocytes.

The extent of type III collagen distribution increased in proportion to that of type I collagen as the disease progressed.

In an absorption test, positive staining was diminished after antibody absorption in each staining.

DISCUSSION

The findings of this immunohistochemical study were: 1) all of the components examined were observed in the fibrotic regions, ie, no primary deficit was seen for any of the components examined, 2) fibronectin was found throughout the course of the disease, 3) laminin and type IV collagen in the fibrotic lesion were not restricted to the myocyte membrane, and 4) the extent of type III collagen distribution increased in proportion to that of type I collagen.

In control hamsters, fibronectin was distributed in the peri- and endomysial spaces. These findings are consistent with those reported by Casscells et al²⁰ who used the ABC method in rat heart. Similarly, the staining patterns and localization of laminin and type IV collagen, and those of type I and III collagens were the same as those

reported by Chapman and Eghbali²¹ and Speiser et al²² respectively, who used an immunofluorescent method in mouse and human hearts. Furthermore, the distribution of each component examined in the skin, liver and kidneys was similar to that reported previously^{15–19}. The immuno-positive staining for each component was diminished after antibody absorption by the corresponding purified component. These results of the control procedures indicate that the findings obtained by staining in the present method are suitable for analysis.

As stated above, a primary deficit of extracellular matrix components has been implicated in hereditary heart diseases⁸. In this study, we found that all of the components examined were present in various fibrotic lesions; we found no lack of these components.

Fibronectin was observed throughout the course of the disease. In experimental myocardial infarction in rats, fibronectin appeared earlier than type I and III collagens^{20,23} and disappeared later during healing, ie, when scar formation was complete. The same earlier appearance and later disappearance of fibronectin have been reported in wound healing^{24,25}. These recent studies of fibronectin indicate that it could play an important role in the initiation of fibrotic changes²⁶. Therefore, fibronectin may contribute to the continuous fibrotic changes seen here in cardiomyopathic hearts. Furthermore, the presence of fibronectin indicated that the fibrotic changes were not complete; ie, this was immature, on-going fibrosis, which may cause ventricular dilation in this pathological condition.

Laminin and type IV collagen, which are both components of the basement membrane, appeared in the lesions and were not limited to the myocyte membrane. The same distribution has been reported in human DCM²⁷. These two components contributed not only to basement membrane-related fibrosis but also to replacement and wide endomysial fibrotic lesions not related to the basement membrane, indicating that both components also contribute extensively to the fibrotic changes in the hearts of cardiomyopathic Syrian hamsters.

Laminin, a major component of the basement membrane, is a large molecule of 850

kDa, with many functional domains, including a binding site to type IV collagen. Therefore, laminin has many biological functions⁶ including the stimulation of cell attachment, migration, growth, and differentiation. Biological studies have suggested that laminin plays a key role in the organization of the matrix assembly⁵. Furthermore, laminin is the first matrix protein to be detected during embryogenesis⁵. Similarly, laminin appears immediately beneath the neo-epidermis in early phases of the wound healing process^{28,29}. These findings indicate that laminin contributes to the initiation of collagen matrix formation.

Both type I and III collagens were present in fibrotic lesions until the end-stage of the disease. The present results indicate that both of these collagens play a major role in the fibrosis that occurs in cardiomyopathic Syrian hamsters. As stated above, the extent of type III collagen distribution increased in proportion to that of type I collagen throughout the course of the disease.

The extent of type III collagen distribution did not decrease during the observation period. In experimental myocardial infarction, polarized light microscopy revealed that green fibers (indicating type III collagen) decreased when fibrosis was complete and were replaced by orange fibers (indicating type I collagen)³⁰. Larjava et al³¹ using in situ hybridization, studied infrabony inflammatory lesions and found that a gradual switch from type III to type I collagen gene expression occurred in chronic inflammatory lesions. Although the present immunohistochemical method did not provide accurate quantitative information regarding the volume of type I and III collagens, the continuous increase in the distribution of type III collagen, together with the findings for fibronectin and laminin, indicate that fibrotic changes occurred throughout the course of the disease in cardiomyopathic hamsters.

Type I collagen has the greatest tensile strength among the collagen subtypes^{32,33}. There was no direct evidence of the replacement of type III collagen by type I collagen in this study. In fact, the continuous increase in the extent of the distribution of type III collagen suggests the lack of its replacement by type I collagen, which, in turn, suggests a relatively weak framework.

This may be a mechanism of ventricular dilation³⁴.

The increased distribution of type I and III collagens became apparent in the necrotic stages, before the hypertrophic stage. In spontaneous hypertensive rats, in contrast, fibrotic changes occur concomitantly with the progression of myocyte hypertrophy, and myocardial collagen content decreases when blood pressure is normalized³⁵. In pressure-overloaded rat myocardium induced by renal artery or aortic constriction, increased type I and III collagen deposition have also been reported to occur concomitantly with myocyte hypertrophy³⁶⁻⁴². Myocardial fibrosis induced by isoproterenol administration also progresses in association with myocyte hypertrophy⁴³. This different timing of type I and III collagen deposition indicates that, in cardiomyopathic hamsters, fibrosis was not related to myocyte hypertrophy in the early stage.

Several limitations are apparent in this study. First, there was no quantitative analysis of each component, as noted above. However, sequential changes in localization and in the extent of the distribution of each component were analyzed. Within each specimen, only the localization and extent of distribution were compared. Second, since the hypertrophic and dilation stages could not be clearly distinguished, we could not analyze the separate components in these stages. Third, other extracellular components, such as type V collagen and proteoglycans, were not examined.

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