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Development of Duchenne-Type Cardiomyopathy

Morphologic Studies in a Canine Model

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The development of cardiac lesions was studied in xmd dogs aged from 1 day to 6 years. Cardiac lesions were not present in dystrophic dogs up to 3 months of age. Foci of mineralization were first seen at 6.5 months. A 1-year-old dog had foci of myocyte hypercontraction. Linear and anastomosing fibrosis was present in all dogs 1 year of age or older, most prominently and most consistently within the subepicardial region of the left ventricular (LV) free wall, the LV papillary muscles, and the right ventricular (RV) aspect of the septum. Ultrastructurally, endomysial fibrosis, decreased myofibrillar density, and prominence of mitochondria were consistent features. Severe degenerative changes were present in two dogs and included prominent intracytoplasmic myelin figures, lipid droplets, and lipofuscin. Immunocytochemical studies of an affected dog confirmed the absence of dystrophin in LV myocardium. Characteristic late-onset cardiac lesions, similar to those occurring in Duchenne dystrophy, are a consistent feature of canine X-linked muscular dystrophy (CXMD). (Am J Pathol 1989, 135:671–678)

Duchenne muscular dystrophy (DMD) is the most common and the most devastating form of inherited muscular dystrophy in men. The disease is an X-linked recessive disorder, affecting approximately 1 in 3000 to 4000 male births.[1] The disease is characterized by ongoing degeneration and regeneration of skeletal muscle that leads to replacement of muscle by connective tissue and fat.[2] Patients are wheelchair-bound by their early teens, and usually die in the 3rd decade.[1]

In addition to the profound skeletal muscle lesions, a distinctive cardiomyopathy has been recognized in DMD patients. Cardiac lesions characterized by subepicardial fibrosis, particularly of the posterobasal portion of the left ventricle (LV), occur in later stages of the disease[3–6] and are apparently progressive.[7–9] Although respiratory failure has been the most common cause of death in DMD patients, virtually every DMD patient eventually develops evidence of cardiac lesions.[3,7,9] With improved patient management and prolonged patient lifespan, cardiac failure has become an increasingly important cause of death, with close to 40% of deaths due to cardiomyopathy.[10] Becker muscular dystrophy is a milder form of DMD, with defects in the same gene.[11] Although skeletal muscle lesions are mild compared with those occurring with DMD, severe cardiomyopathy develops in the later stages of the disease in a large percentage of patients.[9]

Recently the gene responsible for DMD, and its protein product, dystrophin, have been identified, and dystrophin has been found to be most abundant in skeletal, cardiac, and smooth muscle.[12] Two animal models, the mdx mouse[13,14] and the xmd dog[15,16] have been shown to lack dystrophin, and are therefore considered genetic homologues of DMD. Severe skeletal muscle necrosis occurs in the neonatal period in both the mouse[17,18] and the dog.[19] However, canine X-linked muscular dystrophy (CXMD) results in progressive overt weakness,[19] whereas the murine disorder results in little or no apparent weakness.[17,18] Immunohistochemical studies have shown that immunoreactive dystrophin is absent from mdx myocardium,[13] and left ventricular lesions have been reported.[20] Reports of histologic changes have not been consistent, however,

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Figure 1. *Cross-section of formalin-fixed heart from a 1-year-old dystrophic dog. There are multiple mineralized foci in a papillary muscle and irregular pale areas within the septum.*

as one study found no cardiac abnormalities,[18] and death due to cardiac failure has not been reported. In the dog, previous reports of single cases describe mineralized foci in the heart.[21,22] Clinical studies of the canine disorder showed that severe cardiac insufficiency occurs,[19] and echocardiographic and electrocardiographic studies showed that almost all xmd dogs older than 6 months have evidence of cardiac lesions (N. S. Moise and B. A. Valentine, 1989, unpublished observations). In this report we document the consistent occurrence of a spontaneous cardiomyopathy in the xmd dog, and present the results of light and electron microscopic and immunohistochemical studies of cardiac muscle obtained during studies designed to characterize the disease process in an xmd dog breeding colony. The clinical signs in two dogs (ages 1 and 6 years) were reported previously.[19]

Materials and Methods

Animals

All dystrophic dogs were part of an xmd breeding colony, and were the descendents of a single affected male.[16] Hearts from 19 dystrophic dogs aged 1 day to 11 weeks were studied and compared with hearts from ten normal littermates of similar ages. Hearts also were studied from older dystrophic dogs aged 6.5 months (one), 1 year (two), 1.5 years (one), and 6 years (one). Three unrelated dogs aged 9 months, 10 months, and 6 years were studied as controls. One dystrophic dog aged 1 year had severe respiratory insufficiency due to inhalation pneumonia, and was markedly dehydrated before it was killed. The 6-year-old dog had been in congestive heart failure for 6 months. Both dogs had severe ventricular hypomotility, as determined by echocardiography. Dogs that did not die naturally were killed with an overdose of pentobarbital followed by exsanguination.

Gross Examination and Light Microscopic Studies

Hearts were removed immediately after death and immersion fixed in 10% buffered formalin. After fixation entire hearts were cross-sectioned, and the thickness of the LV, RV, and septal walls was measured at the level of the base of the valves. For histopathologic examination samples were taken from the atria and auricular appendages, and from the septum, RV, and LV at several levels (a total of 13 to 17 sections per dog). Sections were embedded in paraffin, and 6-μm sections were stained with hematoxylin and eosin (H&E). Masson trichrome stain was used on sections from dogs aged 6.5 months or older.

Electron Microscopic Studies

Formalin-fixed tissue was obtained from the subepicardium of the midportion of the left ventricular free wall of

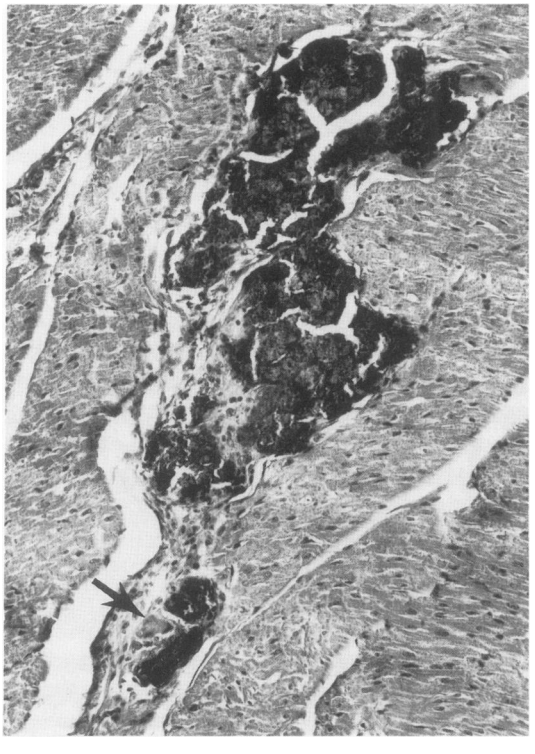

Figure 2. *Acute lesion with focal myocardial mineralization in the left ventricular myocardium from a 6-month-old dystrophic dog. There are associated macrophages and a giant cell (arrow) (H&E, ×125).*

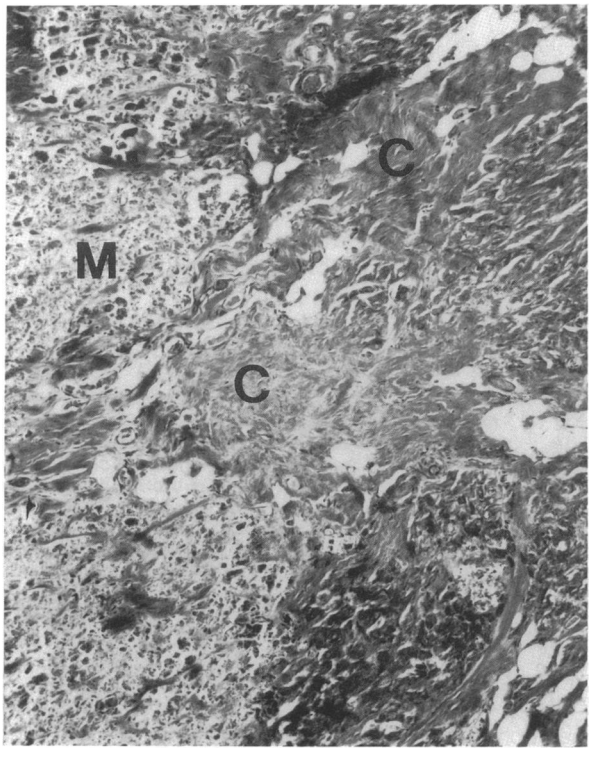

Figure 3. *Large area of myocardial mineralization (M) surrounded by dense connective tissue (C) in the papillary muscle from a 1-year-old dystrophic dog (Masson trichrome, ×35).*

the four dogs aged from 1 year to 6 years, and from the 10-month-old and 6-year-old control dogs. After mincing, specimens were postfixed in osmium tetroxide, dehydrated in alcohols, and embedded in epoxy. Sections 1 μm thick were stained with toluidine blue, and thin sections were mounted on grids, stained with uranyl acetate and lead citrate, and examined on a Philips 201 transmission electron microscope (Phillips Electron Instruments, Eindhoven, The Netherlands).

Immunohistochemistry

Sections of LV papillary muscle were removed immediately after death from a 1-year-old dystrophic dog and a 12-week-old unrelated normal dog. Sections were snap-frozen in isopentane cooled in liquid nitrogen, and 4-μm sections were cut on a Reichert cryostat and stained with either sheep anti-mouse dystrophin (60 kd antigen) antiserum (courtesy of Drs. L. M. Kunkel and E. P. Hoffman, Boston, MA) or normal sheep serum. Immunostaining was performed using the streptavidin-biotin technique (Zymed Histostain SP kit, San Francisco, CA). After incubation in primary or nonimmune serum, sections were incubated in biotinylated rabbit anti-goat IgG, followed by incubation in peroxidase-conjugated streptavidin. Diaminobenzidine was the chromagen, and sections were lightly counterstained with hematoxylin. Normal canine skeletal muscle served as a positive control.

Results

Gross Pathology

No gross abnormalities were present in dogs 11 weeks of age or younger or in any control dogs. In the 6-year-old dog pale streaks were present on the epicardial surface of the ventricles, particularly the apical portion of the LV, and there was thinning of the LV wall in the most severely affected areas. In cross-sections the pale foci extended a few mm into the underlying myocardium, and large areas of the LV free wall were pale and firm. At some levels pale foci were also present in the RV aspect of the septum. Focal white, chalky areas of mineralization were visible on cross section in the dogs aged from 6.5-months to 1.5-

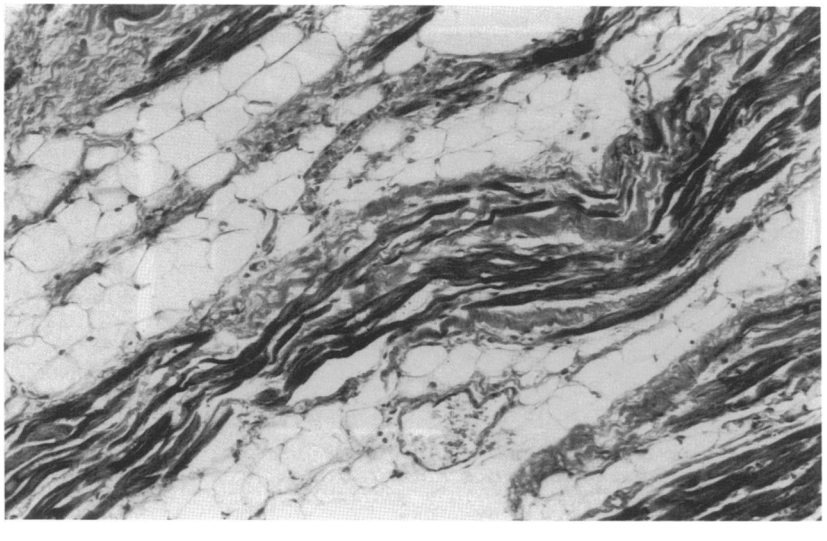

Figure 4. *Fat and connective tissue infiltration of the subepicardial region of the left ventricular myocardium from a 6-year-old dystrophic dog (Masson trichrome, original magnification ×115).*

Figure 5. *Foci of acute necrosis in the left ventricular myocardium from a 1-year-old dystrophic dog with severe pneumonia.* **A:** *Discrete focus of myocardial hypercontraction and degeneration (original magnification ×110).* **B:** *Longitudinal section showing formation of contraction bands and dissolution of myofibrils (original magnification ×360). (Masson trichrome.)*

years, and involved the LV papillary muscles, septum, and LV free wall. Grossly visible mineralization was most severe in the 6.5-month-old dog and in a 1-year-old dog (Figure 1). Other than the focal areas of thinning and mild cardiomegaly noted in the 6-year-old dog, the wall thickness measurements and the overall size of the hearts were within normal limits.

Light Microscopic Findings

No histologic cardiac lesions were present in any dog 11 weeks of age or younger or in controls. All dogs 6.5 months of age or older had prominent lesions within the myocardium. The 6.5-month-old dog had relatively few lesions, and focal dense, basophilic areas of mineralization were the predominant finding. Lesions were most severe in the LV papillary muscle and apical LV free wall, but were also present in the septum and left atrium. The mineralized foci had very little associated connective tissue, and macrophages and foreign body-type giant cells were present (Figure 2). In the LV papillary muscle a large area of myocardium was replaced by loose connective tissue and proliferating vessels. Foci of mineralization were present at the edge of this lesion. Myocytes in adjacent areas were attenuated, vacuolated, and frequently surrounded by a thin rim of connective tissue.

In the 1- to 1.5-year-old dogs lesions were much more widespread, and linear and anastomosing bands of connective tissue were prominent. These linear areas of fibrosis were often circumferential in the LV and septum, and the connective tissue varied from loose fibrovascular tissue to more dense, relatively acellular collagen. Fat in-

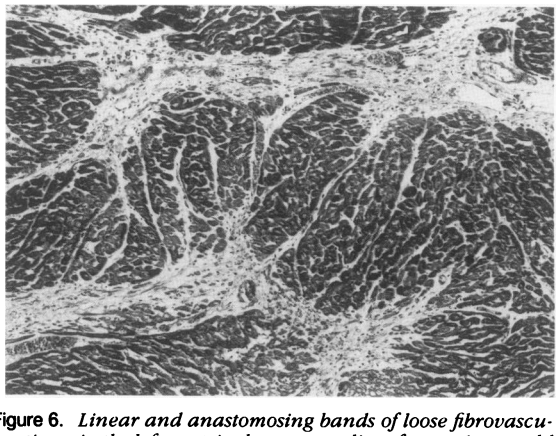

Figure 6. *Linear and anastomosing bands of loose fibrovascular tissue in the left ventricular myocardium from a 1-year-old dystrophic dog. Note the predominantly perivascular pattern of fibrosis (Masson trichrome, ×50).*

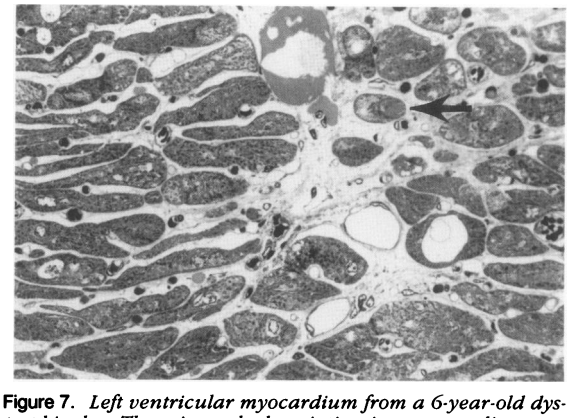

Figure 7. *Left ventricular myocardium from a 6-year-old dystrophic dog. There is marked variation in myocyte diameter, endomysial fibrosis, and fat infiltration. Myocytes are frequently vacuolated, the sarcoplasm is diffusely granular, and one cell contains a large intracytoplasmic fat droplet (arrow) (1-μm section, toluidine blue stain, ×240).*

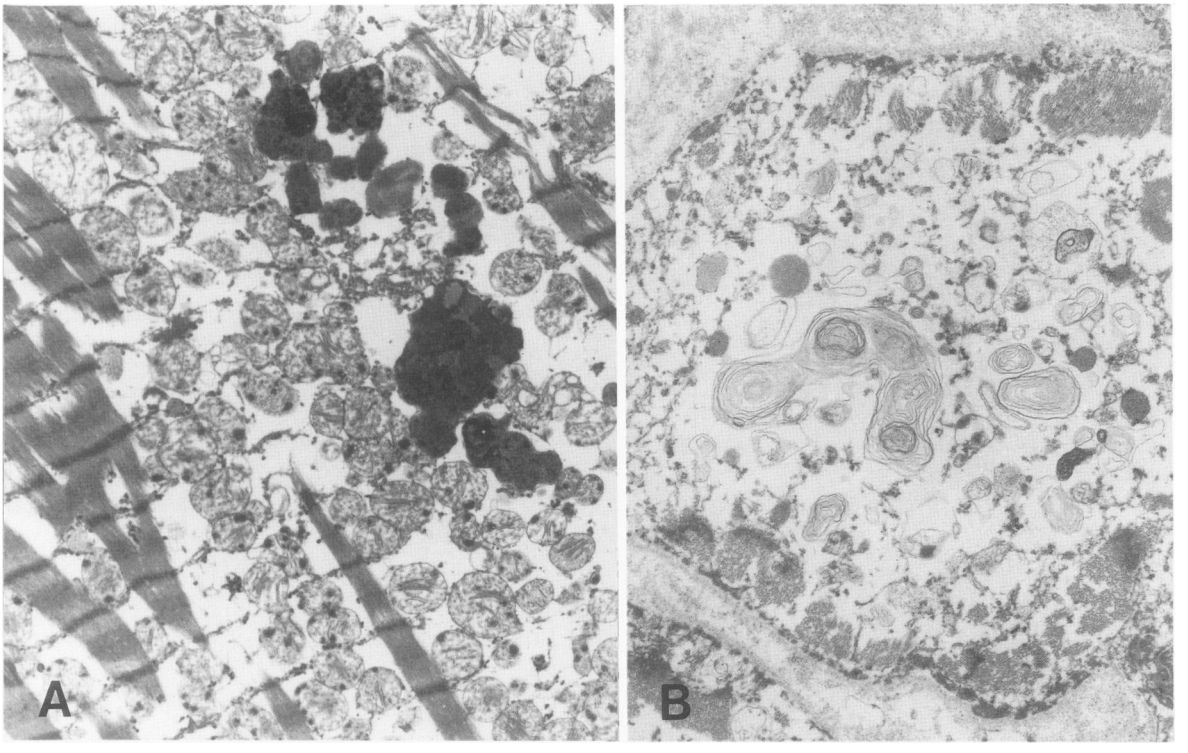

Figure 8. *Ultrastructural findings in the left ventricular myocardium of a 6-year-old dystrophic dog.* **A:** *Increased numbers of mitochondria with loss of myofilaments and aggregates of lipofuscin (×6750).* **B:** *Small diameter degenerate myocyte with prominent central loss of myofilaments and accumulation of myelin figures. The endomysium has markedly increased collagen (×12600). (Uranyl acetate and lead citrate.)*

filtration was uncommon. Mineralized foci were present, but associated macrophages were less common, and all foci were surrounded by dense connective tissue (Figure 3). Myocardial lesions were most consistently present, and were most severe in the LV papillary muscles, in the subepicardial region of the LV free wall in the area extending from the midventricle to the apex, and in the right ventricular half of the septum. In two dogs lesions were present in all areas of the heart, including the atria and RV free wall, but in the latter the changes were much less pronounced than those in the LV and septum.

In the 6-year-old dog there was marked connective tissue and fat infiltration of most areas of the myocardium, often in a striking subepicardial pattern (Figure 4). Areas involved, in decreasing order of severity, were the LV, LV papillary muscles, septum, RV, and both atria. All connective tissue was dense and mature. Foci of mineralization were less common, and were very small compared with those seen in younger dogs. All areas of mineralization were surrounded by dense connective tissue, and there was no evidence of macrophage infiltration. Scattered hypercontracted degenerating myocytes were present adjacent to areas of fibrosis.

Although in all dystrophic dogs myocytes within fibrotic areas were frequently attenuated and sometimes vacuolated, large foci of acute necrosis were present only in the 1-year-old dog with severe pneumonia and dehydration. In this dog, multiple discrete foci of swollen, hypercontracted, and fragmented myocytes were present at several levels of the septum and LV free wall (Figure 5). With Masson's trichrome stain the affected cells were a distinctive dark red color and often had pale centers. Small numbers of macrophages and neutrophils were present in and around many of these foci.

In all dogs areas of fibrosis and mineralization were frequently perivascular, and anastomosing bands of connective tissue often appeared to extend from vessel to vessel (Figure 6). Mild vascular changes were present in the 1.5- and 6-year-old dogs, consisting of focal endothelial proliferation in two to three small arteries in each dog. There was occasional mild, patchy fibrosis within the smooth muscle wall of coronary veins and arteries in all dogs, although vascular lumina were not apparently compromised. In the 6-year-old dog there was focal thinning and connective tissue replacement of the smooth muscle in the wall of one intramural artery.

In 1-μm sections there was increased variation of myofiber size in all dystrophic dogs, and increased endomysial and perivascular connective tissue. The sarcoplasm was frequently granular and sometimes vacuolated. Vacuolated and hypertrophic myocytes were common in the 6-year-old dog, and in this dog there was prominent inter-

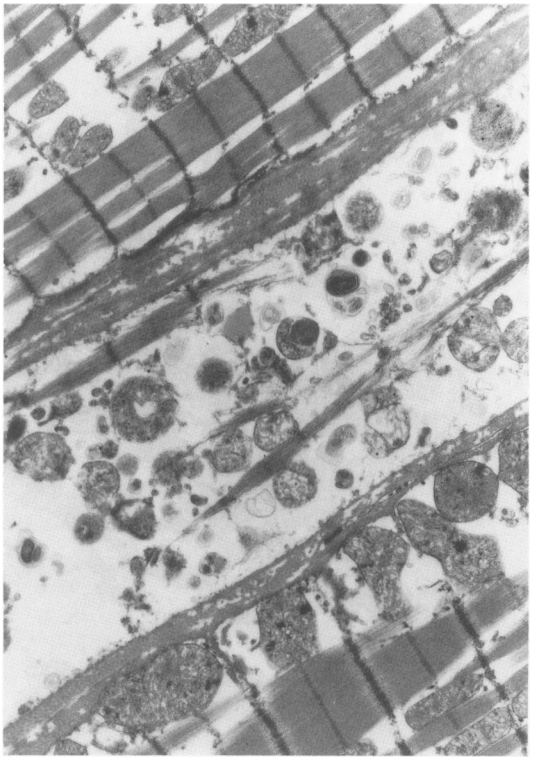

Figure 9. *Ultrastructural findings in the left ventricular myocardium from a 1-year-old dystrophic dog. Degenerating myocytes, one of which shows almost total loss of myofilaments with degenerating mitochondria, lipid, and myelin figures (Uranyl acetate and lead citrate, ×8400).*

stitial adipose tissue and intracytoplasmic lipid droplets (Figure 7).

Electron Microscopic Findings

Increased endomysial collagen was evident in all dogs, and there were scattered myocytes with attenuation of myofilaments and clustering of mitochondria. Ultrastructural abnormalities were most common and severe in the two dogs that were shown to have ventricular hypomotility. In these dogs there were many myocytes with increased numbers and increased size variation of mitochondria and prominent degenerative changes, including myelin figures, increased lipid droplets, autophagic vacuoles, and lipofuscin (Figure 8). Attenuated myocytes with prominent loss of myofilaments were present in many densely fibrotic areas. Some of these cells also had degenerative changes similar to those described above. There were scattered hypercontracted cells, most commonly in the 1-year-old dog in which acute necrosis was detected in paraffin sections. In this dog small diameter myocytes were present in which the sarcoplasm consisted of a few patches of myofilaments, prominent lipid droplets, myelin figures, and mitochondria (Figure 9). Although the formalin fixation employed was not ideal, similar changes were not detected in control samples. Although membrane structure was difficult to assess, no overt sarcolemmal abnormalities were noted. Z band morphology was not altered. Intercalated disks were often irregular in dystrophic dogs, but were otherwise structurally normal.

Immunohistochemistry

In the normal 12-week-old dog stained with sheep anti-mouse dystrophin there was staining of the sarcolemma in all cardiac myocytes. Staining of the dystrophic myocardium was uniformly negative (Figure 10).

Discussion

The light microscopic features of the cardiomyopathy of Duchenne dystrophy are well characterized.[4–6,23] The most striking and characteristic feature noted in hearts obtained at autopsy is subepicardial fibrosis, which is most severe in the posterobasal portion of the LV free wall.[4–6] Frankel and Rosser[6] mapped the development of myocardial fibrosis in eight patients, and proposed that the earliest changes occurred in the posterobasal portion of the LV free wall, with progressive involvement of adjacent portions of LV free wall and the RV aspect of the septum. Other studies identified degenerative changes in LV papillary muscles as well as in the free wall.[24] The striking myocardial fibrosis seen in xmd dogs in this study was virtually identical to that seen in Duchenne patients. These changes were most severe in the 6-year-old dog with con-

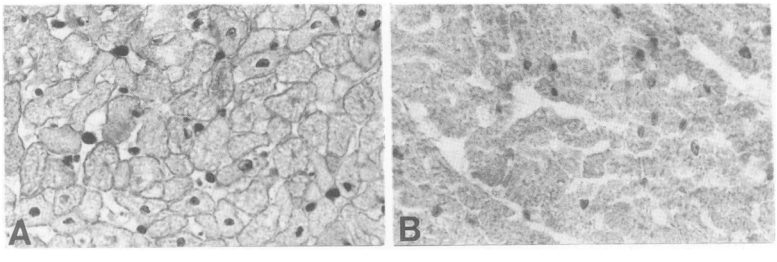

Figure 10. *Immunostaining of normal* (A) *and dystrophic* (B) *canine myocardium with sheep anti-mouse dystrophin. Dystrophin is present in the myocardial sarcolemma of the normal dog, but is completely absent in the dystrophic dog (Streptavidin-biotin technique, Meyer's hematoxylin counterstain, original magnification ×430).*

gestive heart failure, but were present in all dogs 1 year of age or older.

In contrast to the many light microscopic studies, there are only a few reports of ultrastructural lesions in DMD hearts. Autopsy studies showed loss of thick and thin filaments, Z band alterations, dilated sarcoplasmic reticulum, and accumulation of mitochondria.[24,25] These features were also noted in a recent report by Wakai et al,[26] in which both autopsy samples and RV endomyocardial biopsies were studied. In addition, these authors noted increased sarcoplasmic lipofuscin and lipid droplets, even in very young patients with no evidence of cardiac dysfunction. In the dog, endomysial fibrosis and increased variation in myocyte size were the most common ultrastructural findings. All dystrophic dogs had increased numbers of variably sized myocardial mitochondria, and all displayed some degree of myofibrillar attenuation. None of these changes were present in similarly processed control hearts. Degenerative lesions were severe in the two dogs with decreased ventricular contractility. In these animals there were many myelin figures, as well as severe loss of myofilaments and increased intracytoplasmic lipid droplets and lipofuscin.

From these studies it appears that in dystrophin-deficient cardiomyopathy myocardial fibrosis is preceded by myocyte necrosis and, at least in the dog, prominent mineralization. The degree of mineralization decreases with age, most likely due to clearance by macrophages and the degree of fibrosis increases. Myocyte degeneration is apparently an ongoing process that results in gradual replacement of the myocardial tissue by connective tissue. Myocardial fibrosis can eventually result in loss of contractility and congestive heart failure.

Certain regions of the myocardium appear to be particularly prone to develop degenerative lesions. In the dog, early, focal lesions involve primarily the LV papillary muscles and LV free wall, with less severe lesions in the septum and left atrium. Over time, lesions progress and involve large areas of the LV free wall, from the midventricle to the apex and the RV aspect of the septum.

Lesions in the heart, although similar in some ways to those in skeletal muscle,[2] differ substantially in age of onset and severity. Cardiac lesions are a relatively late clinical manifestation of both DMD[3,8,9] and CXMD, and degenerative lesions are mild compared with those of skeletal muscle. Although vascular lesions are uncommon in both DMD[6,27] and CXMD, the striking perivascular distribution of fibrotic lesions in the hearts of dogs with CXMD is noteworthy. It may indicate a peculiar sensitivity of cardiac myocytes in those areas to injury, or it may implicate vascular factors in the pathogenesis of the cardiomyopathy.

The massive acute necrosis seen in the dog with severe pneumonia was an unexpected finding. Similar lesions were seen in one dystrophic dog subjected to anesthesia and open-chest cardiac ^{31}P nuclear magnetic resonance (NMR) studies (B. A. Valentine and M. Osbakken, 1988, unpublished observation). Although similar acute degenerative lesions do not appear to have been reported in Duchenne patients, some investigators noted sudden cardiac failure in DMD patients after illness with a common cold.[26] These observations suggest that stress may incite massive myocardial damage.

The function of dystrophin, the gene product of the DMD locus, and the mechanism by which dystrophin deficiency results in cardiac and skeletal muscle degeneration, are not yet known. Sequence data on the protein,[11] its localization at the plasma membrane of cardiac, skeletal, and smooth muscle cells,[13] and its linkage to an integral membrane glycoprotein[28] suggest that it is a cytoskeletal protein, possibly involved in membrane stabilization. Although lack of dystrophin in myocardial cells may prove to be directly responsible for degeneration of cardiac myocytes, the possibility of dysfunction of vascular smooth muscle cells cannot be ruled out. It is hoped that further prospective studies of the cardiac lesions in the dog model will lead to a better understanding of the pathogenesis of cardiomyopathy in dystrophin-deficient muscular dystrophy.

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