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Abnormal Fibrillin Metabolism in Bovine Marfan Syndrome

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Bovine Marfan syndrome is a disorder that closely resembles human Marfan syndrome in its clinical signs and pathological lesions. The similarities between the human and bovine diseases suggest that similar metabolic defects could be responsible. Although indirect immunofluorescent assays for fibrillin in skin biopsies did not distinguish affected cattle from control animals, cultures of skin fibroblasts of affected animals were distinguished from normal, unrelated control animals and normal half-siblings on the basis of fibrillin staining. After 72 to 96 hours in culture, stained with anti-fibrillin monoclonal antibody 201, hyperconfluent fibroblast cultures of affected cattle had less immunoreactive fibrillin than control cultures, and the staining pattern was granular rather than fibrillar. Under similar culture conditions, normal bovine aortic smooth muscle cells produced large amounts of immunoreactive fibrillin, but smooth muscle cells from a single affected cow showed markedly less fibrillin staining. In pulse-chase metabolic labeling experiments with [^{35}S]cysteine, dermal fibroblasts from 6 affected calves, incorporated far less fibrillin into the extracellular matrix than control cells. These findings are similar to those reported in human Marfan syndrome, and they suggest that the bovine Marfan syndrome, like the human disorder, is caused by a mutation in fibrillin, leading to defective microfibrillar synthesis. (Am J Pathol 1993, 142:803–810)

Marfan syndrome is a life-threatening, autosomal dominant genetic disease affecting approximately 1 in 10,000 individuals worldwide.[1] The disease affects many organ systems, but the most severe manifestation is a progressive dilation and ultimate dissection of the proximal aorta. The pleiotropic nature of the condition has suggested a defect in a connective tissue protein, but until recently little progress had been made in defining the molecular basis of the disease.

Fibrillin is the major 350-kd protein constituent of the 8 to 10–nm microfibrils found in many tissues,[2] including the elastic fibers of the aorta and skin and in the ciliary zonule, tissues often affected in Marfan syndrome. The fibrillin gene has been mapped to human chromosome 15,[3] a locus that has been tightly linked to the Marfan locus.[4,5] Seventy percent of human fibrillin complementary DNA sequence has been published,[6] fibrillin clones have been used to link tightly the fibrillin locus to the Marfan locus;[7,8] and one mutation in the fibrillin gene has been identified in 2 patients with de novo mutations that result in the Marfan phenotype.[8] Studies of fibrillin synthesis and processing by dermal fibroblasts from individuals with Marfan syndrome have demonstrated abnormalities in the way that cells from affected individuals process fibrillin.[9] These findings all strongly suggest that the genetic defects responsible for human Marfan syndrome lie within the fibrillin gene.

Research on the diagnosis and treatment of Marfan syndrome has been hampered by the lack of any animal models. In 1990, Besser and others described a group of related cattle with many of the major clinical manifestations of the human disease.[10] The usefulness of this model depends in part upon demonstration of metabolic and etiological similarities between the human and bovine syndromes and upon the ability to breed the animals successfully. To

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determine the relationship between human Marfan syndrome and the bovine model, we examined fibrillin expression in skin, dermal fibroblast cultures, and aortic cell cultures using indirect immunofluorescence. We found decreased fibrillin staining in cultured dermal fibroblasts and aortic smooth muscle cells, similar to findings in cells from people with Marfan syndrome. Decreased incorporation of fibrillin into the extracellular matrix was confirmed by pulse-chase metabolic labeling experiments in affected animals.

Materials and Methods

Bovine Marfan Syndrome Model

The bovine Marfan syndrome model has been described previously.[10] Briefly, 7 related calves (3 males and 4 females) had musculoskeletal, ocular, and cardiovascular lesions that led to the diagnosis of Marfan syndrome based upon clinical criteria used in humans.[11] The calves exhibited tall stature, thin extremities (dolichostenomelia), joint hypermobility, displaced lenses (ectopia lentis), and aortic root dilatation when compared to age-matched, unaffected half-siblings. One castrated male calf died of spontaneous aortic rupture at the age of 16 months, and a 2-year-old female was euthanized in extremis with heart failure due to dissecting aneurysms of the aorta and the pulmonary artery.

All probands were the offspring of a single phenotypically normal purebred Limousin bull. All dams were cross-bred animals unrelated to the bull. For these reasons, the bovine syndrome was thought to be the result of a germ-line mutation in the bull and an autosomal dominant inheritance pattern was proposed.[10] Further evidence for autosomal dominant inheritance was the birth of 3 of 4 affected offspring from superovulation of a single affected cow bred to unrelated purebred bulls of the Jersey breed (Figure 1).

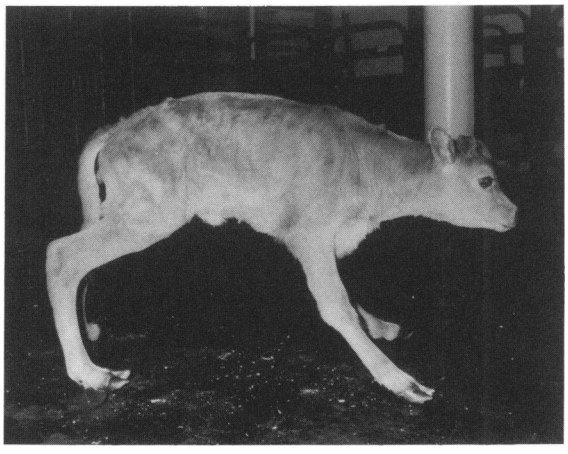

Figure 1. *Bovine Marfan syndrome. This calf was the product of superovulation/embryo transfer of a 2-year-old, affected female bred to an unaffected, unrelated bull of the Jersey breed. The calf was diagnosed as affected with bovine Marfan syndrome based upon hypermobility of distal joints, ectopia lentis, and aortic dilation. Aortic root diameter at 1.5 weeks of age was 3.6 cm.*

Tissue Specimens

Skin Biopsies

All biopsies were taken by sterile technique under local anesthesia. Skin of the posterior thigh was shaved, scrubbed with Betadine solution, and swabbed with alcohol. Lidocaine was infused subcutaneously. Five mm biopsies were obtained using a biopsy punch, and biopsy sites were closed with a single suture. One biopsy from each animal was immediately frozen in liquid nitrogen and stored at −70 C until assayed. A second biopsy was immediately placed in Hanks' balanced salt solution with 200 units penicillin G, 200 µg streptomycin, and 120 µg tylosin per ml and used to start primary dermal fibroblast cultures (see below).

Aortic Smooth Muscle Cells

Cells were obtained from the tissues of an 8-month-old female bovine abattoir specimen and from an 8-month-old affected steer that was humanely destroyed for the purpose of initial characterization of the model. Both tissue specimens were taken as full thickness sections of the aortic arch, 5 to 6 cm distal to the aortic valve. Tissue specimens were collected within 30 minutes of death, placed in media as described above, and used to start primary cultures.

Tissue Cultures

Primary cultures were initiated as previously described[12] with some modifications. Briefly, 1 mm minces of dermis were plated into 6 well culture dishes (Falcon, Lincoln Park, NY) in Dulbecco's minimal essential medium (DMEM) with 30% fetal calf serum (FCS, Hyclone, Logan, UT), 20 mmol/L Hepes and 500 units penicillin G, 500 µg streptomycin, 1.25 µg/ml fungizone, and 60 µg tylosin per ml and allowed to grow for 10 to 14 days. At 75% confluency, cells were detached with trypsin and passaged into 25-cm^2 tissue culture flasks (P1). After P2, FCS concentrations were lowered to 10%. For fibrillin immunofluorescent studies on dermal fibroblasts, all cell lines were below P10. For aortic cell studies, cell

lines were between P2 and P12. To verify the identity of aortic smooth muscle cells, indirect FA staining was performed using monoclonal anti-α smooth muscle actin (Sigma Chemical Co., St. Louis, MO) and polyclonal anti-VWF (Sigma) to rule out the presence of endothelial cells.

Indirect Immunofluorescence

Indirect immunofluorescence staining was performed as previously described with minor modifications.[13,14] Skin sections were embedded frozen in Tissue Tek (Miles Laboratories, Naperville, IL), and 8-μ sections were cut on a cryostat at −25 C. Fibroblasts and aortic media cells were plated into 4-chamber microscope slides (LabTek, Naperville, IL) at 1 or 2×10^5 cells/chamber. Incubation medium was DMEM with 10% FCS, 20 mmol/L Hepes, 100 units penicillin G, 100 μg streptomycin, and 60 μg tylosin per ml. Cells were incubated for 2 to 10 days at 37 C in 5% CO_2. All tissue sections and chamber slides were coded before staining so that the diagnosis of affected versus normal was unknown during processing.

The primary antibody was monoclonal antibody 201, which was previously shown to be specific for fibrillin and to cross react with bovine fibrillin in tissue sections.[2] Antibody source was either hybridoma supernatant, which was used without dilution, or IgG isolated from ascites fluid, which was diluted 1:100. The second antibody was rabbit anti-mouse IgG+IgM conjugated to fluorescein isothiocyanate (Sigma). Tissue sections and cell cultures were air dried for 30 minutes fixed in cold acetone for 10 minutes, and incubated in a humidified chamber with primary antibody for 3 hours at room temperature. Slides were then washed gently in 3 changes of phosphate-buffered saline and incubated with second antibody (1:50) for 1 hour. After 3 washes in phosphate-buffered saline, slides were incubated in 0.00025% propidium iodide for 5 minutes to stain nuclei. Slides were then again washed in phosphate-buffered saline, and coverslips were mounted in buffered glycerol with 0.1% p-phenylenediamine.

Each group of tissue sections or cell cultures included samples from at least 1 known normal animal. Passage number varied from 2 to 12, but groups of cells plated for each experiment were within 2 passage numbers. One of us with extensive experience in fibrillin immunofluorescence has shown that fibrillin expression is relatively insensitive to passage number (LYS, unpublished data). Slides were examined and scored as normal or affected based upon fibrillin immunofluorescence by at least 1 and often 2 individuals, without knowledge of the clinical status (single blind study). Slides were examined and photographed using a Zeiss fluorescence microscope equipped with a M35 camera.

Preparation and Electrophoretic Analysis of Fibrillin

Synthesis, secretion, and processing of bovine fibrillin by dermal fibroblasts was evaluated as previously described for human cells.[9] To radiolabel synthesized proteins, 250,000 dermal fibroblasts were plated in 35-mm dishes (Corning, Corning, NY) and allowed to attach and spread for 72 hours in Dulbecco-Vogt modified Eagle medium (DMEM) supplemented with 10% FCS (Irvine Scientific, Irvine, CA). The medium was replaced with DMEM lacking FCS and cysteine but supplemented with sodium ascorbate (50 μg/ml) and incubated for 2 hours. Then the cells were pulsed with [^{35}S]cysteine (50 μCi in 0.15 mls) in a cysteine-free medium supplemented with ascorbate. The medium was removed, the cells were washed extensively with DMEM and then incubated in the presence of DMEM supplemented with 50 μg/ml ascorbate for up to 10 hours. The medium was collected and the cell layer washed in DMEM and then harvested in 50 mmol/L TrisHCl, pH 8.0, 1% NP40, and 1 mmol/L phenylmethyl-sulfonyl fluoride. After the cell lysate was aspirated from the dish, the material remaining on the dish was scraped into the same buffer with a rubber policeman, and the insoluble proteins were collected by centrifugation at 10,000 *g* for 5 minutes at 4 C. Proteins were dissolved in sample buffer containing sodium dodecyl sulfate and β-mercaptoethanol,[15] separated by electrophoresis in 4% acrylamide slab gels containing sodium dodecyl sulfate, and localized by autoradiography. ^{14}C-labeled molecular weight standards (Amersham, Arlington Heights, IL) were used to estimate molecular weights of labeled proteins.

Immunoprecipitation

Bovine fibrillin was identified in aliquots of medium and cellular lysate by immunoprecipitation using monoclonal antibody 201[2]. The antigen-antibody complex was precipitated with protein G Sepharose (Pharmacia, Milwaukee, WI) as previously described.[9]

Results

Immunofluorescence

Skin biopsies from 6 affected cattle, 2 unaffected half-siblings, and 4 unrelated, unaffected, age-

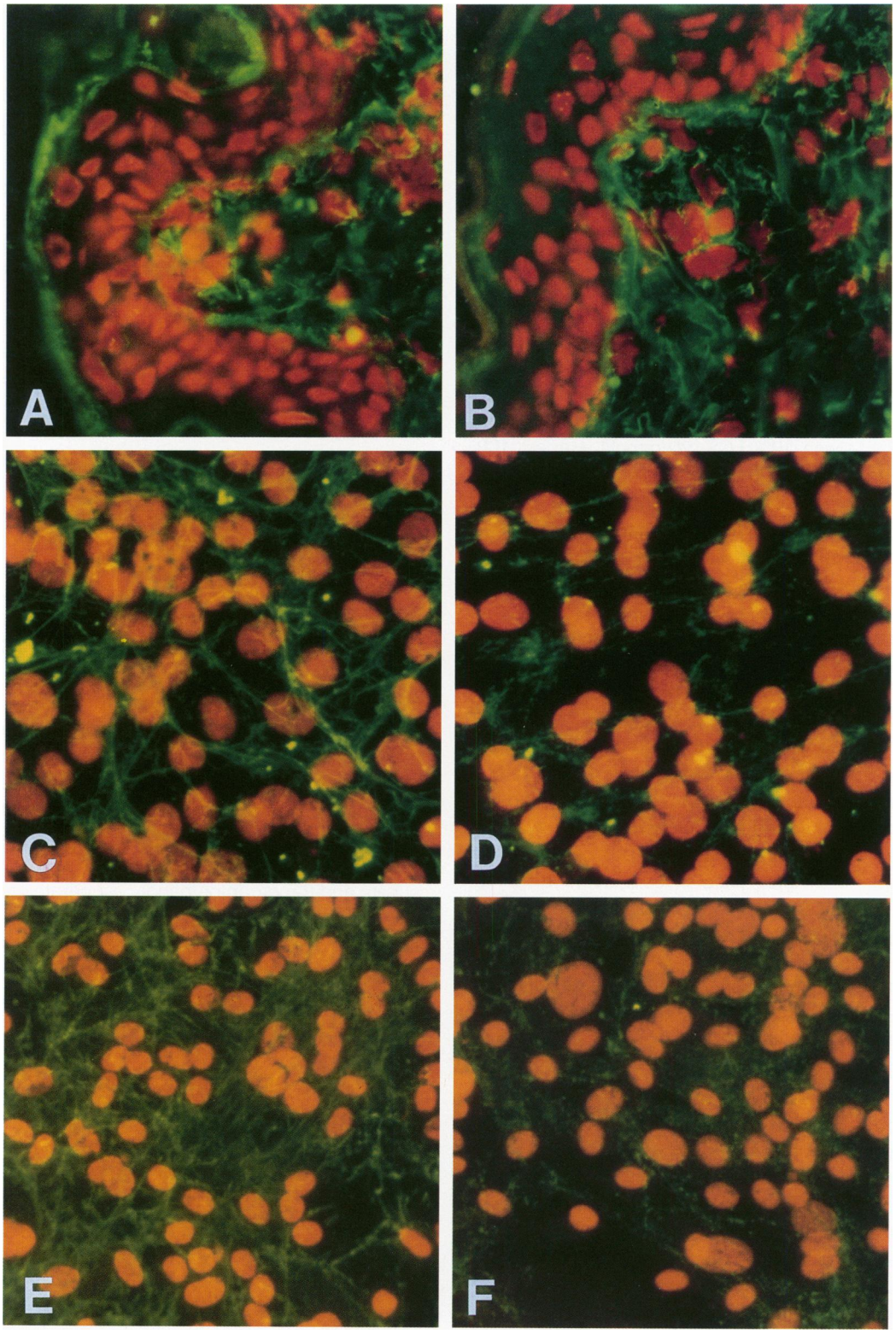

Figure 2. *Indirect immunofluorescence of skin* (A *and* B), *dermal fibroblast* (C *and* D), *and aortic smooth muscle cell cultures* (E *and* F) *from normal cattle* (A, C, E) *and cattle affected with Marfan syndrome* (B, D, F). *Skin sections were from unaffected* (A) *and affected* (B) *full siblings (original magnification ×400). C and D: Bovine fibroblasts, 2 × 10^5/ml, incubated 4 days (original magnification ×400). E and F: Bovine aortic smooth muscle cells, 1 × 10^5/ml, incubated 6 days (original magnification ×400). Affected cattle had normal to slightly decreased IFA for fibrillin in skin but markedly decreased fibrillin expression in tissue cultures of dermal fibroblasts and aortic smooth muscle cells.*

matched cattle were incubated with anti-fibrillin monoclonal antibody 201, stained with fluorescein isothiocyanate–conjugated anti-mouse IgG, and examined by fluorescence microscopy for immunoreactivity to fibrillin without knowledge of clinical status (Figure 2, A and B). All animals, whether clinically affected with Marfan syndrome or not, had a similar pattern of fibrillin reactivity, which was comparable to the staining of normal human skin.[3] Subtle differences seen in photographed specimens were not distinguished in blind studies.

Examination of cultured dermal fibroblasts for fibrillin immunoreactivity, however, did allow distinction between affected and control animals (Figure 2, C and D). Monoclonal antibody 201 detected significant fibrillin immunoreactivity at 4 to 6 days in culture in control fibroblasts, whether from unaffected related animals or from unrelated normal animals (Figure 2C). Cultured dermal fibroblasts from all 7 affected cattle showed less immunoreactive fibrillin than control cultures examined at the same time. Fibrillin in fibroblast cultures of affected cattle displayed a granular staining pattern, rather than the fibrillar pattern seen in normal human and cattle fibroblast cultures (Figure 2D).

Cultured aortic smooth muscle cells from one affected and one unrelated normal animal were also examined for fibrillin immunoreactivity (Figure 2, E and F). The normal bovine aortic cells produced large amounts of fibrillin, as demonstrated by immunofluorescence. In contrast, cells from 1 affected animal had markedly less fibrillin immunoreactivity, with a granular pattern similar to that seen in fibroblasts from affected animals.

Synthesis and Secretion of Fibrillin by Bovine Fibroblasts

The monoclonal antibody 201 was used to immunoprecipitate fibrillin from the media of bovine fibroblasts continuously labeled for 20 hours with [^{35}S]cysteine (Figure 3). A large protein with molecular mass of approximately 320 kd was precipitated, and that protein co-migrated with the extracellular-processed form of human fibrillin (Figure 3, arrow). The major protein immunoprecipitated from the cell lysate following a 30-minute label with [^{35}S]cysteine had an approximate molecular mass of 350 kd; a minor second band with a molecular mass of about 300 kd was also precipitated (Figure 3, double arrows).

After a 30-minute pulse, several high-molecular-weight proteins, including fibrillin, were labeled with [^{35}S]cysteine in bovine dermal fibroblast cultures.

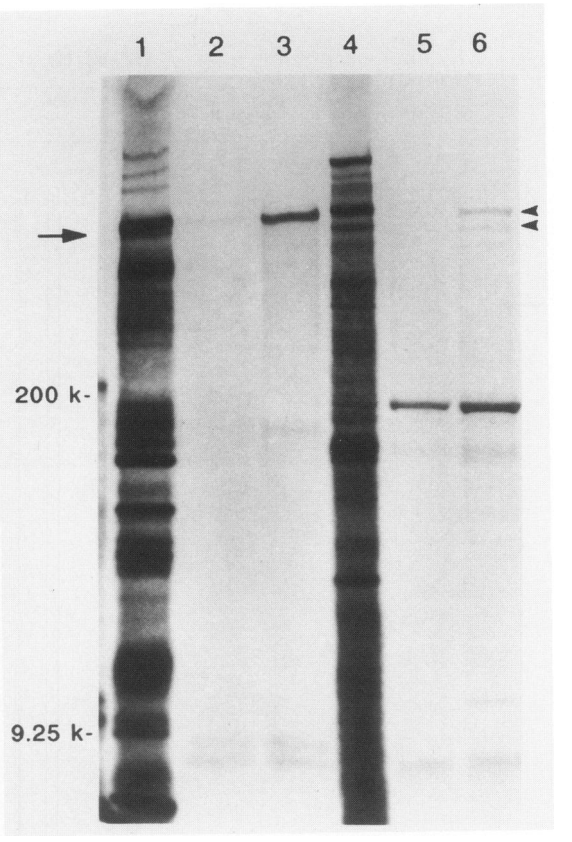

Figure 3. *Identification of bovine fibrillin. Human or bovine fibroblasts were radiolabeled either by incubating with [^{35}S]cysteine continuously for sixteen hours* (lanes 1 *to* 3) *or pulsing the cells with [^{35}S]cysteine for 30 minutes* (lanes 4 *to* 6). *The secreted proteins* (lanes 1 *to* 3) *or the intracellular proteins* (lanes 4 *to* 6) *were harvested and fibrillin was isolated by immunoprecipitation.* Lanes 1 *and* 4 *contain the total secreted and intercellular proteins made by bovine fibroblasts, respectively.* Lanes 2 *and* 5 *are precipitates without antibody present.* Lanes 3 *and* 6 *are precipitated with 100 μl of antibody present. Bovine fibrillin immunoprecipitated from the medium as a single band (arrow). Two bands are immunoprecipitated from intracellular proteins (arrowheads). The larger of the 2 bands is compatible with the larger, intracellular form of fibrillin. A 200-kd protein is non-specifically precipitated from the intracellular proteins with and without antibody present. Molecular weight markers are shown.*

Although intensely labeled after the pulse, the intracellular pool of fibrillin diminished significantly by 2 hours and was completely gone by 8 hours (Figure 4). Fibrillin secreted into the culture medium had a slightly faster migration, presumably due to extracellular proteolytic processing. The lower-molecular-weight, extracellular form of fibrillin appeared in the matrix as early as 8 hours. Only the proteolytically processed, extracellular form of fibrillin was deposited in the matrix (Figure 4).

Bovine fibroblasts from 6 affected and 3 normal half-siblings were studied in a single blind protocol for defects in fibrillin synthesis or processing (Figure 5). Autoradiographs were examined by one of us (DMM), and calves diagnosed as affected or normal without prior knowledge of clinical status. After a

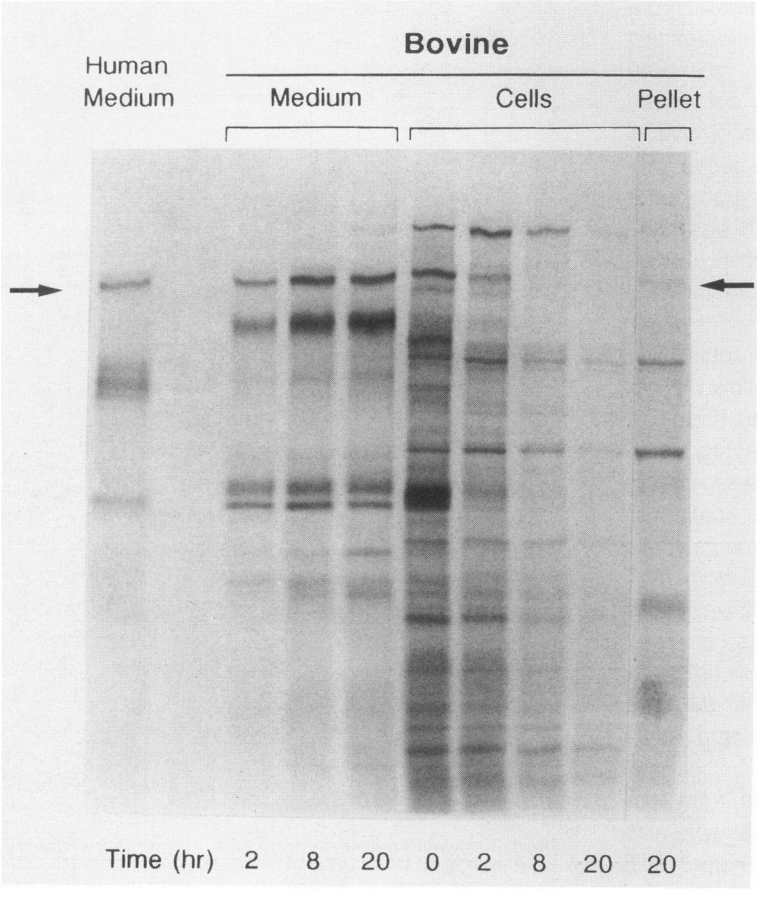

Figure 4. *Synthesis and secretion of fibrillin by cultured human and bovine fibroblasts. Human fibroblasts were labeled continuously for 16 hours with [^{35}S]cysteine and the medium harvested. Bovine cells were incubated for 30 minutes in the presence of [^{35}S]cysteine (0 time) and then chased up to 20 hours in the absence of label. The medium, intracellular (cells), and matrix (pellet) proteins were then harvested. Human fibrillin (arrow) co-migrates with bovine fibrillin. As with human fibrillin,[9] bovine fibrillin was synthesized as a precursor. Secretion began within 2 hours and virtually all fibrillin had been secreted by 8 hours. At 20 hours, fibrillin appeared as a single band in the cell associated matrix (pellet). The molecular weight of this band is compatible with the smaller, extracellularly processed form of fibrillin also present in the medium.*

30-minute pulse, of [^{35}S]cysteine, cell strains from affected or control animals made similar amounts of fibrillin and secreted it from the cell within 10 hours. In the cell strains from control animals, the lower-molecular-weight form of fibrillin was present in the matrix by 10 hours. In the cell strains from affected animals, fibrillin was not found in the insoluble cell–associated proteins after 10 hours of chase. The

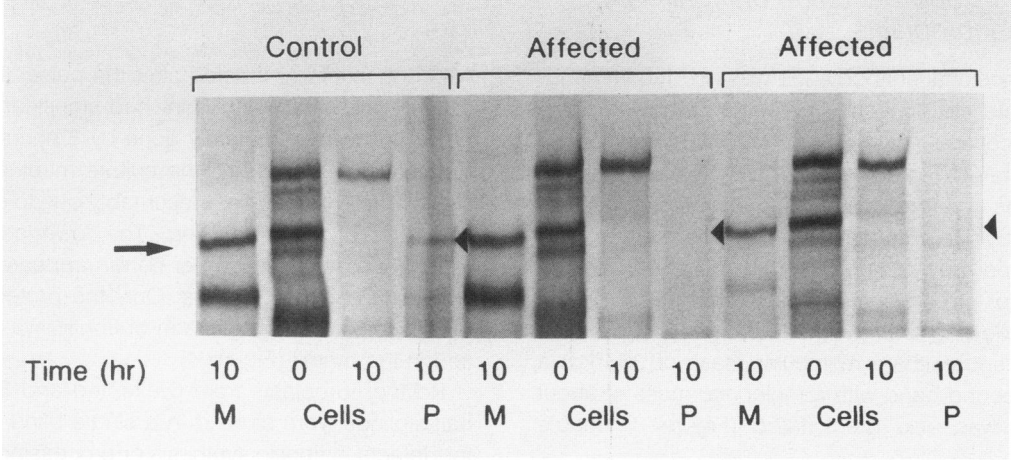

Figure 5. *Abnormal incorporation of fibrillin into the cell-associated matrix by dermal fibroblasts in bovine Marfan syndrome. 2.5 × 10^5 cells were incubated with [^{35}S]cysteine for 30 minutes, and the cells were then chased 10 hours with unlabeled medium. The medium (M), cell lysate (Cells), and cell-associated matrix (P) were then harvested and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The amount of fibrillin (arrow) made by all 3 cell strains was similar, but only the control cells deposited an appreciable amount of fibrillin in the matrix (arrowheads). Other anonymous protein bands are of equal density in control and affected samples.*

source of each of the nine cell strains were identified correctly by this assay.

Discussion

Bovine Marfan syndrome is similar to human Marfan syndrome, in that affected cattle have joint hypermobility, dolichostenomelia, ectopia lentis, and aortic dilation and die of aortic dissection. The defect in fibrillin metabolism that we found in bovine Marfan syndrome is similar to one reported in human Marfan patients.[9,13] These data suggest that the genetic defect in the bovine syndrome, as in the human syndrome, resides within the fibrillin gene.

Bovine fibrillin has been previously shown to have a tissue distribution and antigenic make-up similar to the human molecule.[2] We have shown that bovine fibrillin is also expressed in cultures of normal bovine dermal fibroblasts and aortic smooth muscle cells in quantities similar to those seen in cultured human dermal fibroblasts and that fibrillin immunoprecipitated from [^{35}S]cysteine-labeled bovine fibroblasts has a molecular weight similar to human fibrillin. The identity of the 300-kd protein precipitated from bovine fibroblast cell lysate is unknown but may represent either an antigenically related molecule,[7] or proteolytically degraded fibrillin. Pulse-chase metabolic labeling with [^{35}S]cysteine has demonstrated that normal bovine fibrillin is produced, secreted from the cell, and incorporated into the extracellular matrix in a time and manner parallel to that of fibrillin synthesized by cultured human dermal fibroblasts.

Our finding of decreased fibrillin staining in cultures of bovine Marfan dermal fibroblasts in the face of normal staining of skin biopsy specimens is similar to what has been reported in human Marfan syndrome. When skin and dermal fibroblast immunofluorescence was used,[13] 24 of 27 people with clinical Marfan syndrome were correctly identified as affected based upon decreased fibrillin reactivity in skin, dermal fibroblast cultures, or both. Four of those 24 individuals had decreased immunofluorescence in fibroblast cultures only, similar to the results we have found in Marfan cattle. In addition, we found decreased fibrillin immunoreactivity in cultured aortic smooth muscle cells of Marfan cattle; aortic smooth muscle cells from humans with Marfan syndrome have not yet been studied.

Studies of fibrillin synthesis and processing identified metabolic defects in cell strains from human Marfan patients: decreased amounts of fibrillin synthesized, inefficient secretion of fibrillin from the cell, and abnormal incorporation of fibrillin into the extracellular matrix. A few cell strains had no demonstrable abnormalities.[9] Pulse-chase experiments reported here showed that cells from affected calves made normal amounts of fibrillin and secreted it efficiently from the cell but were unable to deposit fibrillin into the extracellular matrix. This defect in cattle is comparable to a common defect found in human Marfan cell strains and is consistent with the pattern of decreased fibrillin immunofluorescence in tissue culture matrix. The correlation of these different metabolic defects with clinical severity and specific mutations in the fibrillin gene requires additional fibrillin sequence data in both the human and bovine syndromes.

Little is understood about how fibrillin aggregates into microfibrils. Presumably molecules of fibrillin polymerize in an orderly fashion in the extracellular matrix to form microscopically visible microfibrils. The mutation in these animals most likely alters the structure of the protein such that either the microfibrils are slow to form in culture, or the microfibrils formed do not express the epitope recognized by antibody 201. Since these animals are heterozygous for the mutation (as indicated by breeding studies), the abnormal fibrillin protein may disrupt aggregation of the product of the normal allele into normal fibrils, as occurs in several forms of osteogenesis imperfecta.[16]

The results of this study support the bovine Marfan syndrome as a bona fide model of the human disease, and strongly suggest that the defect in cattle lies in the fibrillin gene. Comparison of clinical phenotype, fibrillin metabolism, and specific mutations between the human and bovine syndromes will help clarify the pathogenesis of this complex and important genetic disease. Marfan syndrome cattle can provide an important resource for testing new drug therapies to delay progression of aortic dilation and decrease risk of aortic dissection.

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