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Cardiac Myofibroblasts Express Alpha Smooth Muscle Actin During Right Ventricular Pressure Overload in the Rabbit

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A number of changes occur in contractile proteins and mechanical performance of the heart within 2 weeks of right ventricular pressure overload in 8- to 12-week-old rabbits. These changes are accompanied by increases in collagen concentration and the ratio of type I to type III collagen. The purpose of the present study was to evaluate the evolution of these connective tissue changes morphologically and to characterize the interstitial cells that might be responsible. The myocardium is infiltrated by mononuclear inflammatory cells 2 days after banding, accompanied by focal myocyte necrosis. By 7 days, the inflammatory infiltrates subside and the damaged myocytes seen at 2 days are replaced by new collagen and a population of spindle-shaped cells, with ultrastructural features of myofibroblasts. A significant proportion of these cells contain alpha smooth muscle actin by immunohistochemical analysis. At 14 days, there is a large increase in stainable collagen with complex remodeling and reduplication of the collagen fiber network of the interstitium. Alpha smooth muscle actin-containing myofibroblasts persist, but their immunoreactivity appears reduced compared with day 7. The authors hypothesize that the interstitial fibroblasts that acquire smooth-muscle–like features in this model play a critical role in the heart's response to severe and sudden mechanical stress and are at least partly responsible for the changes in connective tissue that occur as a result of pressure overload in this model. (Am J Pathol 1991, 139:207–216)

The heart responds to pressure overload with a coordinated pattern of changes in composition, structure, and mechanical performance required to maintain adequate function.[1–6] These changes include extensive remodeling of the cardiac interstitium involving altered concentrations and proportions of individual components such as the collagens.[4,7,8] These extracellular proteins are thought to play a role in the overall response, based on the importance of the connective tissue matrix to maintaining cell–cell relationships and distributing mechanical stress during and between myocyte contractions.[1,4,5,7–9] The mechanisms of interstitial matrix remodeling, however, and the signals involved remain poorly understood.

Our own studies have identified a series of specific biochemical alterations in connective tissue elements that occur in a rapidly evolving model of ventricular pressure overload in the rabbit.[6] These involve an increase in total collagen and the collagen I/collagen III ratio, with a fall in elastin concentration.[6]

The purpose of the present study was to provide the morphologic basis for understanding the relationship between the remodeling interstitial compartment and the cytoskeletal features of the interstitial cells responsible for the observed changes in connective tissue.

Methods

Pulmonary Artery Banding

The method for producing right ventricular pressure overload in rabbits has been reported.[10,11] Briefly, 9- to 11-week-old male New Zealand White rabbits were obtained from local suppliers and were maintained for 7 days on standard rabbit chow and water *ad libitum* before pulmonary artery banding. The procedure consists of placing a spiral metal wire band around the trunk of the pulmonary artery, resulting in a 66% narrowing of this vessel. In accord with previous reports, no animals developed congestive heart failure after operation.[6,10–12] A total of five

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control animals was compared with five animals at day 2, five at day 7, and four at day 14 after banding.

Tissue Processing

Animals were killed by intravenous injection of 0.8 ml of T-61 euthanasia solution (Hoechst-Roussel Co. Summerville, NJ). Hearts were removed and the atria were dissected from the ventricles before weighing. The right ventricle was immediately sectioned into six 2- to 3-mm strips perpendicular to the apex–base axis. Alternate sections were placed in 50% alcohol for light microscopic studies. Remaining sections were diced to less than 1 mm^2 and processed for ultrastructural evaluation (see below).

Light Microscopic Studies

Sections for light microscopy were processed into paraffin wax, sectioned at 4 μ, mounted on glass slides, and stained with hematoxylin and eosin or the immunoperoxidase method.

Immunocytochemical Analysis

Histologic sections were cut at 4 μ, dewaxed in xylene, and rehydrated through graded solutions of ethyl alcohol in preparation for immunolocalization studies. The avidin-biotin complex immunoperoxidase method (Vector Laboratories, Burlingame, CA) was used for localizing immunoreactivity using a modification of the method of Hsu et al.[13] Primary antibody incubations were performed at 4°C for 12 hours. IgG2A monoclonal antibody without defined specificity and buffer alone were used as antibody and method controls, respectively. Diaminobenzidine (DAB) tetrahydrochloride (Polysciences, Warrington, PA) was used as the chromogen at a concentration of 0.5 mg/ml in trishydroxyaminomethane (TRIS) buffer (pH 7.6) in the presence of hydrogen peroxide (25 μl of 1% H_2O_2/5 ml DAB). Sections were counterstained lightly with Harris' hematoxylin for morphologic orientation, dehydrated, and coverslipped with mounting medium. Counterstain was omitted for some of the photomicroscopy.

Semiquantitative analysis of alpha actin immunoreactivity was performed on paraffin-embedded tissue sections from three randomly selected hearts each from days 2, 7, and 14, using a Bioquant (Meg X) image analyzer (R & M Biometrics, Nashville, TN). Briefly, sections were immunoreacted in the same procedure to insure uniformity of staining. Each heart section was overlaid 100 times with an electronically generated box of a preset area. The box size selected allowed the 100 measurements per section to be overlaid with minimal overlap. The operator (KOL) was blinded to area selection immediately before each overlay, but fields were rejected if any large blood vessels were present. This condition was preestablished to eliminate the substantial contribution of vascular smooth muscle to the analysis. Thus interstitial alpha actin immunoreaction product was measured. A gray-scale–generated threshold, preset and maintained throughout all 900 measurements, was used for quantitation, and lighting was carefully controlled and checked using a standard.

Antibody

A murine monoclonal antibody (hybridoma supernatant) specific for smooth muscle actin[14] was a gift from Drs. Omar Skalli and Giulio Gabbiani (hybridoma culture supernatant). This antibody also was used as ascites fluid from a commercial source (Sigma, St. Louis, MO). Optimal antibody concentration was determined to be approximately 40 μg/ml by serial dilution in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA).

Histochemical Analysis

Experimental and control heart sections from each interval studied were reacted with ammoniacal silver using the method of Gordon and Sweets[15] for visualizing reticulin fibers. Briefly, sections were oxidized in potassium permanganate, bleached in oxalic acid, and sensitized in 2.5% ferric ammonium sulfate before impregnation with ammoniacal silver. Sections then were reduced in 10% aqueous formalin, toned in 0.1% gold chloride, and fixed in 5% sodium thiosulfate. Experimental and control tissues were impregnated at the same time to insure consistency of silver deposition.

Ultrastructural Studies

Tissue Preparation

For routine electron microscopy, heart slices were cut into small pieces (approximately 1 mm^3) and fixed by immersion in one-half strength Karnovsky's fixative (2.5% glutaraldehyde, 1% formaldehyde in 0.1 mol/l [molar] Millonig's phosphate buffer, pH 7.2) for 45 minutes at 4°C. After three 10-minute washes in Millonig's buffer, the pieces were postfixed in 1% osmium tetroxide in 0.15 mol/l Millonigs's buffer for 45 minutes at 4°C. Finally the tissue pieces were dehydrated in graded ethanols and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife and picked up on copper mesh grids. After counterstaining with 2% ethanolic uranyl ac-

etate and lead citrate, the sections were examined in a Zeiss EM 10 electron microscope.

Immunoelectron Microscopic Studies

Heart slices were cut into small pieces (1 mm^3) and fixed by immersion in 3% (para)formaldehyde, 0.1% glutaraldehyde (vacuum distilled) in 0.01 mol/l phosphate buffer, 0.15 mol/l NaCl, pH 7.4 (PBS) for 1 hour at room temperature. Tissue pieces then were rinsed in PBS (3 × 10 minutes) followed by immersion in 0.05 mol/l NH_4Cl in PBS for 30 minutes to block free aldehyde groups. After further rinses in PBS, the tissue pieces were stored overnight in PBS at 4°C. The tissue was dehydrated in ethanols at progressively lower temperature and embedded in Lowicryl K4M[16] at −35°C according to standard protocols.[17] Ultrathin sections were cut with a diamond knife and picked up on parlodion-/carbon-coated nickel grids. Antigenic sites were detected with the protein A–gold technique.[18] Briefly, colloidal gold particles (10 nm diameter) were prepared by the tannic acid/citrate reduction method[19] and complexed with protein A according to standard protocols.[18] For the immunolocalization of alpha smooth muscle actin, sections were inverted on drops of PBS containing 0.5% ovalbumin for 15 minutes, followed by incubation for 18 hours at 4°C on droplets of antibody. After rinses with PBS (2 × 5 minutes each), the grids were floated for 1 hour at room temperature on droplets of protein A-gold (diluted with PBS containing 1% BSA, 0.075% Triton X-100, and 0.075% Tween 20 to yield an absorbance of 0.06 at 525 nm). After rinses with PBS (3 × 5 minutes) and distilled water (2 × 2 min) the grids were air dried and counterstained with 3% uranyl acetate in distilled water and lead acetate (Millonig's second recipe) before viewing in the electron microscope.

Results

As reported previously,[6,10,12] pressure overload led to a rapid and significant right ventricular hypertrophy. There was a near twofold (1.9×) enlargement of the right ventricle expressed as right ventricular weight over total ventricular weight compared with controls over the course of the 14 days of the experiment (not shown). Right ventricular chamber dilatation was also evident.

Light Microscopy

The morphologic changes in the myocardium began by day 2 of pressure overload, and were characterized by mild edema of the interstitium and focal mononuclear cell infiltration with associated myocyte necrosis (Figure 1B). At 7 days, the reticulin stain showed patchy displacement of myocytes by fusiform interstitial cells and collagen (Figure 2C). Fourteen days after overload, there was less apparent displacement of myocytes but increased interstitial collagen by the reticulin method, both in scarlike foci and diffusely throughout the myocardium (Figure 2D). Interstitial fibroblasts were less distinct than at 7 days, but still appeared increased in number.

Alpha smooth muscle actin immunoreactivity in the control rabbit heart was restricted to smooth muscle of the blood vessels (Figure 3A). Interstitial cells did not appear immunoreactive for alpha smooth muscle actin until day 7. At this time, these immunoreactive cells could be seen within areas displacing myocytes (Figure 3C). By 14 days, interstitial cell staining with alpha smooth muscle actin appeared more delicately filamentous than at day 7, and overall, there seemed to be a decrease in the surface area occupied by these cells. Immunoreactive cells were always evident in a patchy distribution (Figure 3D). Semiquantitative analysis (Figure 5) confirmed that the changes in the amount of alpha actin immunoreactivity was increased at day 7 and day 14 compared with day 2 ($P < 0.05$). There was an apparent decrease at day 14, which did not achieve statistical significance (day 7 *versus* day 14).

Electron Microscopy

The control right ventricle contained only rare interstitial fibroblasts. Two days after pressure overload, macrophages and lymphoid cells appeared in the interstitium and an increase in fibroblasts was noted (Figure 4B). By 7 days, there was a proliferation of fusiform cells with prominent rough endoplasmic reticulum, elongated and slightly serrated nuclei, and irregularly arranged microfilament bundles (Figure 4C). These latter bundles were periodically punctuated with dense areas, and the filaments frequently intersected the cytoplasmic membrane at oblique angles. The above-described features correspond to those described for myofibroblasts.[14,20–23] Fourteen days after overload, these interstitial myofibroblasts were still present but their numbers were decreased and overshadowed by collagen accumulation between myocytes (Figure 4D).

Ultrastructural localization of alpha smooth muscle actin paralleled that seen by light microscopy. No immunoreactivity was seen in interstitial fibroblasts before day 7. Highly specific microfilament labeling was seen in myofibroblasts beginning at day 7 after pressure overload (Figure 4). Labeled microfilaments also were identified at day 14 (not shown).

Discussion

We show here for the first time that acute right ventricular pressure overload in the rabbit leads to the appearance

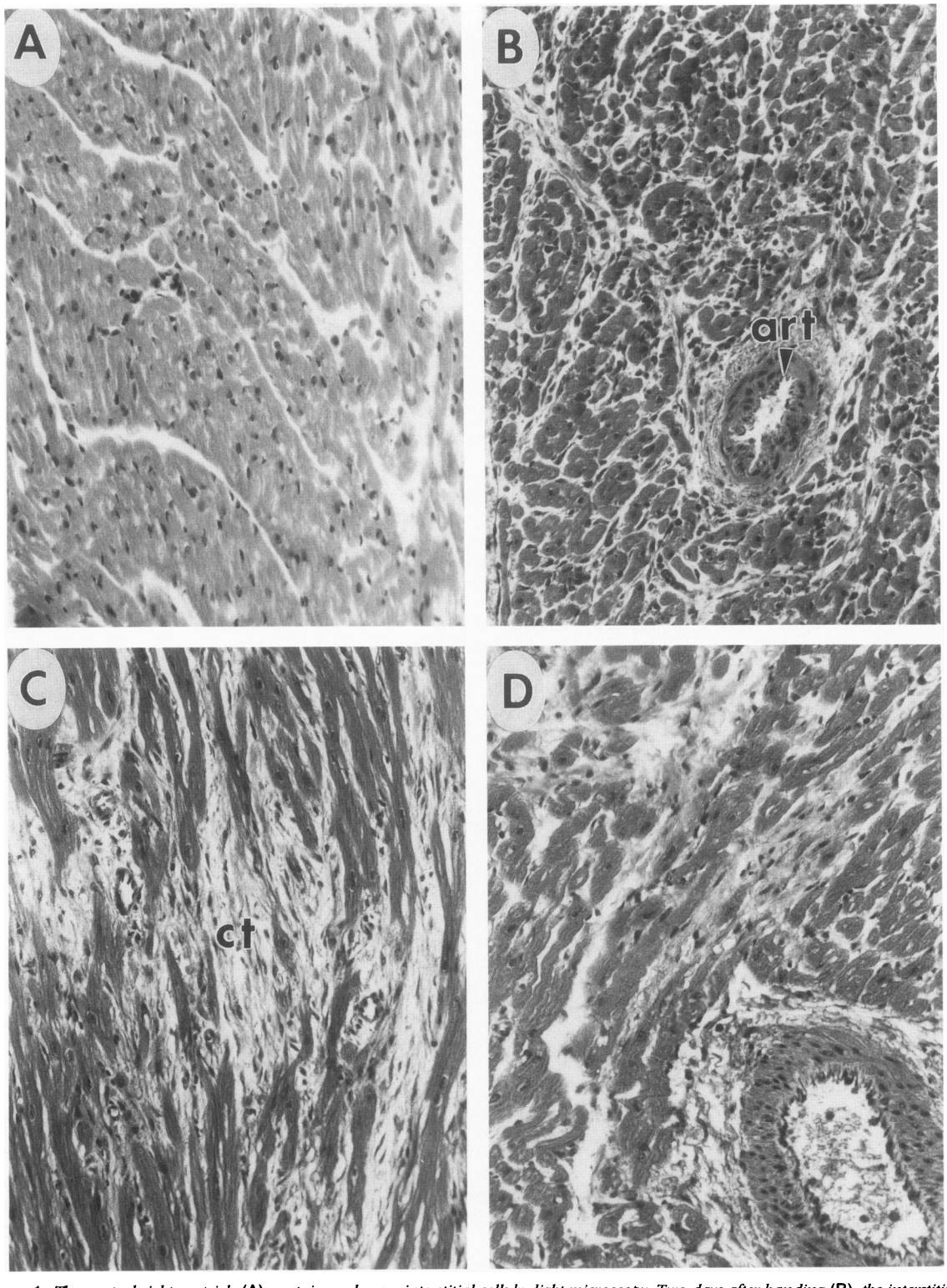

Figure 1. *The control right ventricle* **(A)** *contains only rare interstitial cells by light microscopy. Two days after banding* **(B)**, *the interstitium is focally expanded by macrophages and lymphocytes. A prominent medium-sized arteriole (art) shows increased muscle wall thickness as a proportion of diameter. At 7 days* **(C)**, *loose areolar connective tissue (ct) can be seen displacing myocytes, and inflammatory infiltrates have subsided. By 14 days* **(D)**, *these latter areas are occupied by denser collagen. (H & E stain. Original magnification ×50.)*

of a myofibroblast-like interstitial cell characterized by the expression of alpha smooth muscle actin. The emergence of these cells during a time of rapid connective tissue deposition suggests a role in the extensive remodeling of the interstitial matrix that occurs. The acquisition of alpha smooth muscle actin during this period may

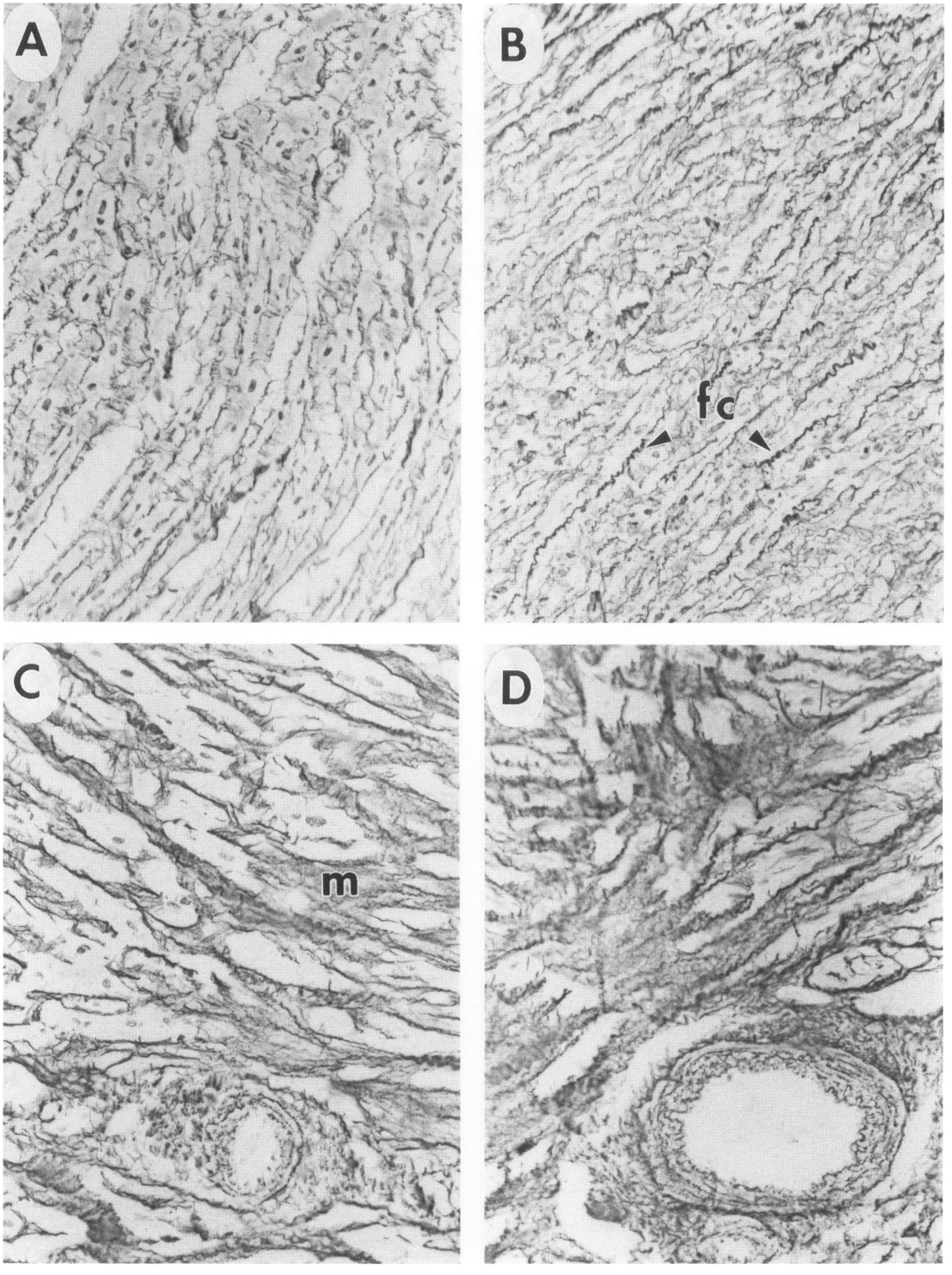

Figure 2. *The reticulin fiber pattern of the normal heart is remarkably symmetric and delicate* **(A)**. *Two days after banding* **(B)**, *the myocardium shows more complexity, and the reduplicated fiber coils (fc) are apparent. By day 7* **(C)**, *the interstitial compartment is expanded markedly with patches of matrix (m) interconnecting regions of myofiber dropout. At 14 days, reticulin fiber density in these areas has increased and these areas resemble a lattice of scar tissue. (Reticulin silver stain, original magnification ×50.)*

serve as a mechanism for tissue contraction, which in effect might serve to reduce the impact of focal injury on the overall function of the myocardium.

At least six actin isoforms exist in mammalian cells.[14,24,25] Beta and gamma actins are found in virtually all cells, whereas alpha-cardiac and alpha-skeletal actins

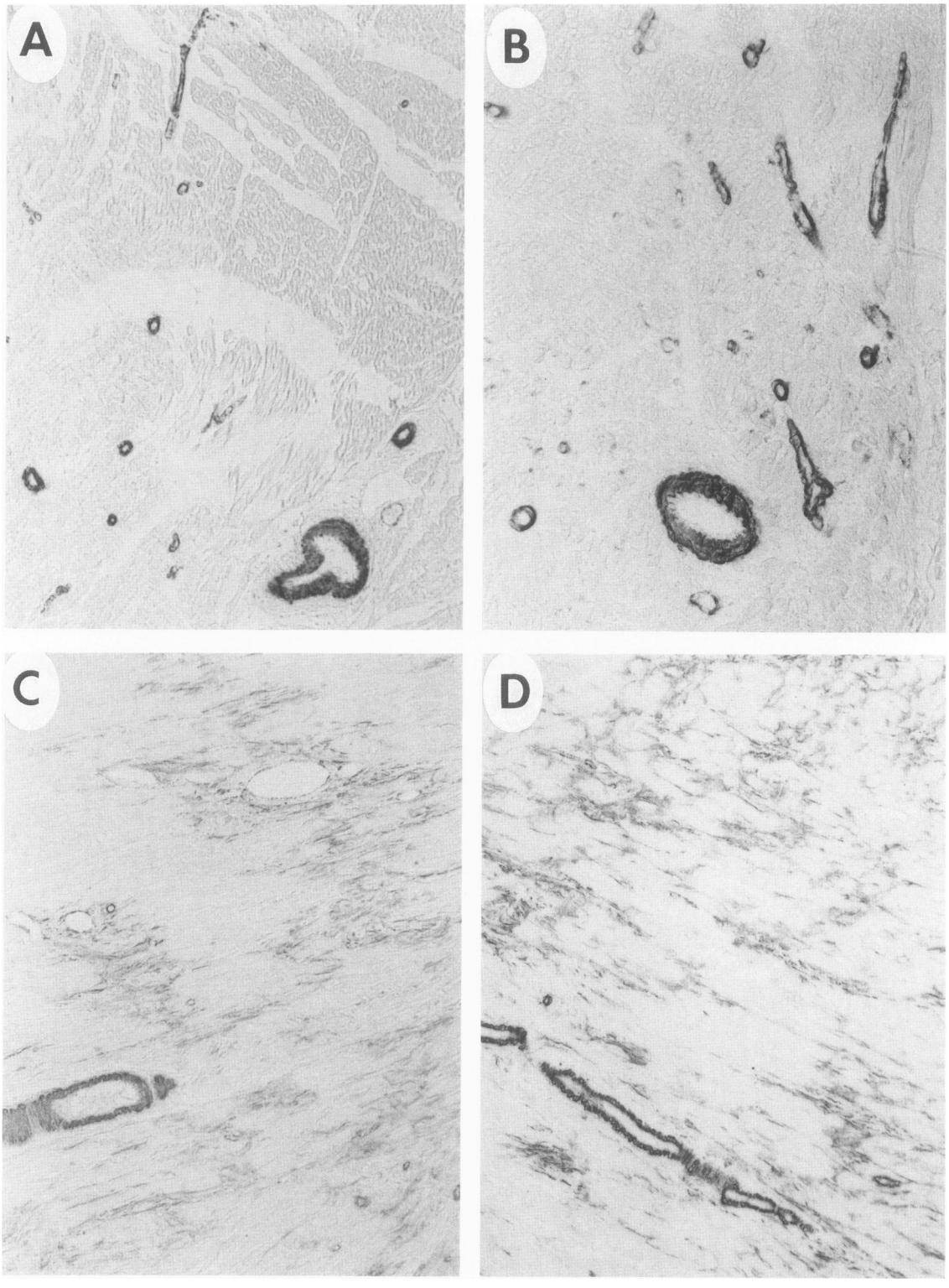

Figure 3. *Alpha smooth muscle actin reactivity in control heart* **(A)** *is confined to the smooth muscle of blood vessels. Two days after banding* **(B)**, *there is an increase in the number of vascular profiles per unit area. Beginning at 7 days* **(C)**, *interconnected patches of immunoreactive spindle cells are seen corresponding to widened areas seen in H & E stains. The character and volume of this reactivity is slightly diminished by day 14* **(D)**. *(Immunoperoxidase stain, original magnification ×50.)*

are thought to be restricted to those two striated muscle types. Two smooth muscle actin isoforms, alpha and gamma, also have been identified. The alpha isoform is now known to occur in several types of putatively contractile nonmuscle cells of intermediate phenotype, including myoepithelial cells and vascular pericytes.[21–25]

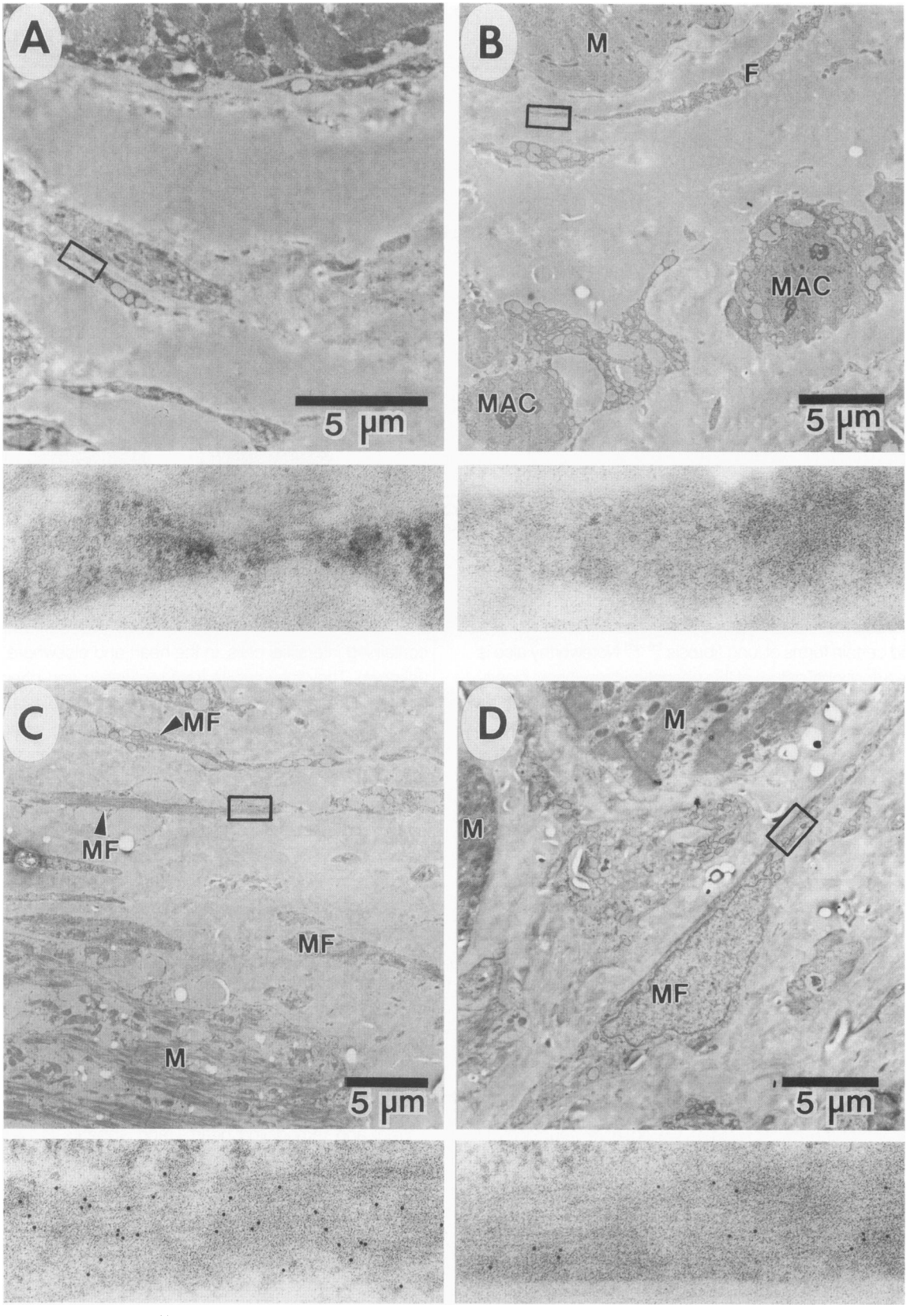

Figure 4. *Control heart* (**A**) *interstitial fibroblasts are few in number and do not contain microfilament bundles or evidence of alpha smooth muscle actin expression. Two days after banding* (**B**), *macrophages and lymphocytes can be seen. Fibroblastlike cells with rare microfilament bundles appear increased within the interstitium, although these do not react with alpha smooth muscle actin* (**B**, *insert*). *At 7 days* (**C**), *alpha smooth muscle actin–positive myofibroblasts with prominent microfilament bundles* (**C**, *insert*) *can be seen in the widened interstitium. At 14 days* (**D**), *myofibroblasts persist but labeling density appears decreased per cell. In addition, more of the spindled interstitial cells resemble true fibroblasts with few microfilament bundles and abundant rough endoplasmic reticulum. (Uranyl acetate-lead citrate.)*

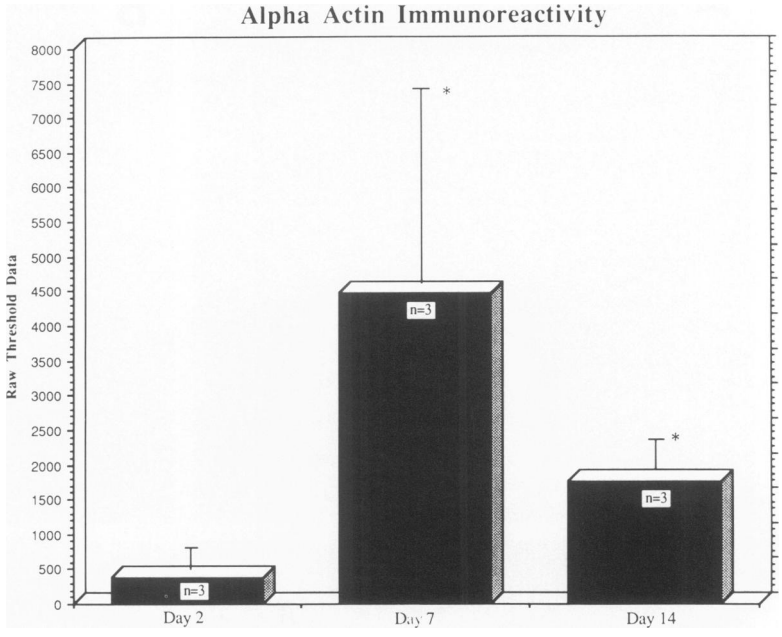

Figure 5. *Semiquantitative analysis of alpha smooth muscle actin immunoreactivity. Three heart sections from each postbanding interval were studied. Columns represent means and standard deviations for each group. Asterisks refer to statistical differences (P = <0.05) for comparisons between day 2 and day 7, and day 2 and day 14. Statistical significance was not reached for the comparison between day 7 and 14. Statistical testing was based on analysis of variance using log transformation of the raw data measurements.*

More recently it has been shown that alpha smooth muscle actin also occurs in a subclass of myofibroblasts that characterize certain types of integument wound repair[21] and certain forms of lung fibrosis.[26–28] Noteworthy also is the transitional nature of this phenotypic response, clearly documented in experimental wound repair[21] and also suggested in the present study.

Our data suggest that the emergence of a population of alpha-actin–positive myofibroblasts is analogous to that seen in other pathologic processes, including those described above. In this regard, it is interesting to note the changes we detected between day 7 and day 14 in our model, a time that also coincides with a period during which there is rapid deposition of collagen and a change in the proportion of types I and III collagen.[6] These changes have been shown to be the result of an increase in collagen synthesis accompanied by increased amounts of collagen mRNA,[29] which return toward normal by day 14 after overload.

Finally the concentration of elastin falls during the 2-week period studied, although there is a net increase in this connective tissue protein.[6] We hypothesize on this basis that the myofibroblasts we observe bear primary responsibility for these changes in the connective tissue matrix, although the specific extracellular matrix synthesis capacity of these cells in the heart remains to be determined.

The emergence of these 'myofibroblasts' in injury has been postulated as a mechanism for effecting tissue contraction.[20] The importance of such wound remodeling can be appreciated in organs such as skin, lung, and heart where structural integrity is directly related to overall organ function. By contraction, the impact of a focal injury on surrounding structures is minimized, thereby reducing the loss in total function.

The origin of the alpha smooth muscle actin-containing interstitial cells, in the heart and elsewhere, is unknown. They may evolve from replication and phenotypic reprogramming of resident interstitial fibroblasts.[21–23,26] Alternatively they could be derived from smooth muscle of the vascular wall or from capillary pericytes. Although not the focus of the present study, our impression gained from inspection of vascular elements within our myocardial sections (Figure 1D) is that vessel size has increased. A more complete quantitative analysis of this phenomenon and additional studies of the cytoskeletal and contractile protein profile of these interstitial myofibroblasts may well provide important clues with regard to their origin.[22]

Of additional importance is the nature of the regulatory signals that are responsible for the observed changes in fibroblast function and phenotype. Two that particularly intrigue us are growth factors and mechanical deformation. A growth factor of particular interest is transforming growth factor beta,[30] which is known to be present in normal and remodeling heart[31] and appears to be involved in the regulation of muscle phenotype.[30,32,33] Mechanical deformation is an equally intriguing potential stimulus, given its proposed role in the myocytic hypertrophy that occurs.[1,3,6] Indeed most recently we have found that mechanical deformation causes proliferation of cultured fibroblasts as well as production of an autocrine growth factor.[34] The role of mechanical deformation in the expression of alpha smooth muscle actin remains to be investigated.

Whether alpha actin is expressed in human right ven-

tricle after pressure overload remains to be determined and is an important target for further study. In such a study, it will be particularly important to compare responses between rapidly evolving overload (eg, diffuse alveolar damage) and chronic, slowly progressive overload (eg, chronic obstructive pulmonary disease).

In summary, our studies in rabbit right ventricular pressure overload suggest an important although as yet clearly defined role for alpha smooth muscle actin-containing myofibroblasts in the adaptive response of the myocardium to this form of stress. We believe that the intimate association of these cells with exuberant connective tissue matrix in the myocardial interstitium provides evidence for an active role for myofibroblasts in connective tissue remodeling of the heart.

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