

Global remodeling of the ventricular interstitium in idiopathic myocardial fibrosis and sudden cardiac death

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KEYWORDS

Arrhythmia;
Collagen;
Extracellular matrix;
Sudden death;
Ventricular fibrillation

OBJECTIVE Characterization of a distinct, and as yet unexplained phenotype of sudden cardiac death (SCD).

BACKGROUND In a subgroup of patients with SCD, postmortem findings are limited to isolated idiopathic myocardial fibrosis (IMF). The absence of confounding factors may facilitate evaluation of the relationship between myocardial fibrosis and ventricular arrhythmogenesis.

METHODS Six patients with IMF were identified from a postmortem, consecutive 13-year series of 270 subjects presenting with SCD. Ventricular interstitial remodeling was assessed quantitatively and qualitatively and comparisons made with 6 age- and sex-matched control subjects who suffered noncardiac death. Myocardial collagen volume fraction and perivascular fibrosis ratio were determined and evidence for inflammatory response and apoptotic cell death was sought. The potential role of transforming growth factor β 1 (TGF- β ₁) in the pathogenesis of IMF was evaluated.

RESULTS Overall myocardial collagen volume fraction was 1.6-fold higher in IMF (mean age 34 ± 4 yrs) vs. controls (mean age 34 ± 4 yrs, $.022 \pm .001$ vs $.013 \pm .001$; $P < .001$). Collagen volume fraction increase was diffuse but disproportionately so in the LV inferior wall (3.4-fold increase; $.035 \pm .005$ vs $.012 \pm .018$; $P < .001$). Perivascular fibrosis ratio was also increased ($.770 \pm .014$ vs $.723 \pm .010$; $P = .007$). There was no evidence of either myocardial inflammatory response or myocyte apoptosis in cases or controls. Expression of TGF- β ₁ was significantly increased in IMF vs controls.

CONCLUSION IMF involves diffuse and heterogeneous remodeling of the ventricular interstitium, with a predilection for the LV inferior wall. TGF- β ₁ is a potential mediator of interstitial remodeling in IMF and SCD.

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Sudden cardiac death (SCD) is a common manifestation of heart disease with an estimated incidence of 180,000 to

450,000 US cases per year.^{1–3} In the overwhelming majority, the acute terminal event is a fatal arrhythmia.⁴ In a subgroup of SCD patients, there are no cardiac structural abnormalities or these are minimal in extent. Consequently, in such patients, potential mechanisms of fatal arrhythmogenesis remain unexplained.^{5,6}

A small but distinct subgroup of patients with SCD have idiopathic myocardial fibrosis (IMF), ie, fibrosis in the absence of other cardiac disease processes. Among autopsy series of SCD, IMF is generally identified in 1% to 3% of

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Table 1 Clinical history and pathologic examination for IMF cases and controls

Patient	Heart wt (gm)	Gross fibrosis	Histologic fibrosis pattern	Clinical information
IMF #1	310	No	Interstitial Replacement	No significant medical history
IMF #2	400	No	Interstitial	No significant medical history
IMF #3	300	No	Interstitial	History of supraventricular tachycardia, normal ECG
IMF #4	260	No	Interstitial	History of seizure disorder and systemic lupus erythematosus
IMF #5	355	Yes	Interstitial Replacement	History of prior ventricular arrhythmia with nondiagnostic work-up
IMF #6	285	No	Interstitial	History of palpitations and hypertension
Mean(SE)	318.3 (20.8)			
Control #1	170	No	None	No significant medical history
Control #2	270	No	None	No significant medical history
Control #3	260	No	None	No significant medical history
Control #4	340	No	None	No significant medical history
Control #5	185	No	None	No significant medical history
Control #6	480	No	None	No significant medical history
Mean(SE)	284.2 (46.6)*			

* $P = .52$ for IMF vs controls

overall cases.⁵⁻⁹ Previously, this pathologic finding has been attributed to the residual effects of myocarditis or age-related degenerative changes.¹⁰⁻¹² However, a detailed characterization of this condition has not been performed.

In general, myocardial fibrosis has been strongly associated with the generation of reentrant tachyarrhythmias and sudden death.^{13,14} The occurrence of IMF in young adults who suffer SCD provides the opportunity to further characterize the potential role of fibrosis in the genesis of fatal arrhythmia, without the confounding effects of other coexisting cardiac conditions, such as coronary artery disease. Overall, myocardial fibrosis is most commonly observed in association with other structural heart disease such as previous myocardial infarction, cardiac hypertrophy, or congestive heart failure. In such conditions, both the occurrence of apoptosis and overexpression of transforming growth factor β_1 (TGF- β_1) have been implicated in myocardial fibrosis.¹⁵⁻¹⁸ We therefore performed a histo-morphometric study in young adults with SCD and IMF and hypothesized that there were quantitative and qualitative differences in the cardiac interstitium when compared to a group of control subjects. We also evaluated the potential roles of apoptotic cell death and TGF- β_1 in the pathogenesis of idiopathic myocardial fibrosis in SCD patients.

Materials and methods

Selection of patients

The Institutional Review Board at the Oregon Health and Science University, Portland, OR approved all aspects of this study. We have previously described a 13-year series (1984-1996) of 270 consecutive patients who presented

with sudden cardiac death.⁶ Of these, 22 cases had evidence of patchy cardiac fibrosis in the absence of post-myocardial infarction scar or any cardiac structural abnormalities specifically associated with SCD. On further examination of these 22 cases, 16 had some form of accompanying cardiac structural abnormality that was nonspecific for SCD, such as left ventricular hypertrophy (LVH) or valvular disease. The remaining 6 cases, classified as IMF, constituted a distinct phenotype among the 270 cases in the autopsy series. In these cases, idiopathic fibrosis was detected in the myocardium without evidence of any additional cardiac abnormalities. Thus, patients with IMF did not have evidence of LVH (heart weight greater than the 95% upper limits of normal or LV wall thickness >15 mm),^{6,19} evidence of prior myocardial infarction, coronary artery luminal narrowing greater than 50%, or any other cardiac structural abnormality including valvular or congenital heart disease. A detailed cardiac histopathological examination was conducted in IMF patients and findings compared to 6 age- and gender-matched control subjects who died of noncardiac causes and underwent a detailed autopsy. The latter subjects were ascertained in a random manner from the Mayo Clinic (Rochester, MN, USA) autopsy archive of patients with structurally normal hearts.²⁰

Quantitative evaluation of ventricular interstitium

Myocardial sections

Hearts archived in 10% neutral buffered formalin were re-examined and full thickness tissue blocks obtained from the mid-ventricular septum; the anterior, lateral, and inferior LV free walls; and the anterior right ventricle (RV). Each specimen was processed routinely and sections of 5 micron

Table 2 Comparisons of collagen volume fraction (CVF) and perivascular fibrosis ratio (PFR) between IMF and controls

	IMF (n = 6)	Controls (n = 6)	P
Collagen Volume Fraction			
Total	0.022 ± 0.001	0.013 ± 0.001	<0.001
Left ventricle	0.021 ± 0.001 [†]	0.012 ± 0.001 [†]	<0.001
Mid-septum LV	0.012 ± 0.001	0.008 ± 0.001	0.003
Mid-anterior LV	0.019 ± 0.002	0.011 ± 0.001	0.005
Mid-lateral LV	0.018 ± 0.001	0.016 ± 0.001	0.373
Mid-inferior LV	0.035 ± 0.005	0.012 ± 0.001	<0.001
Mid-anterior RV	0.026 ± 0.002 [†]	0.018 ± 0.002 [†]	0.001
Perivascular Fibrosis Ratio			
Total	0.770 ± 0.014	0.723 ± 0.010	0.007
Left ventricle	0.791 ± 0.017	0.719 ± 0.011	<0.001
Mid-septum LV	0.782 ± 0.022	0.705 ± 0.023	0.02
Mid-anterior LV	0.810 ± 0.023	0.731 ± 0.022	0.015
Mid-lateral LV	0.732 ± 0.004	0.737 ± 0.023	0.918
Mid-inferior LV	0.757 ± 0.030	0.702 ± 0.023	0.550
Mid-anterior RV	0.763 ± 0.023	0.741 ± 0.023	0.478

*Comparison of IMF vs controls by independent samples *t* test.

[†] *P* < .02, IMF LV vs RV, control LV vs RV.

thickness were prepared from paraffin-embedded tissues were cut 5 microns thick. All sections were de-paraffinized in xylene and rehydrated through graded alcohols prior to staining.

Collagen volume fraction

Sections were stained with picosirius red and viewed under polarized light (40×).^{21,22} Each was divided into four quadrants and four fields selected randomly within each quadrant. A digitized, gray-scale image was obtained and areas of bi-refringent collagen fibrils determined (ImageTool version 2.0, Houston, TX, USA). This step was repeated to determine the area of background myocardium. Collagen volume fraction (CVF) was calculated by dividing the area of collagen fibrils by the sum total area of collagen fibrils and myocardium. CVF for each myocardial section was reported as the average of 16 fields.

Perivascular fibrosis ratio

Sections stained with picosirius red were viewed (10×) to identify intramyocardial arterioles cut in transverse orientation. Three to five arterioles (circumference range 25 to 200 μm) were identified per section. Using ImageTool software calibrated by a stage micrometer, cross-sectional area was manually traced for each arteriole (area A) with a separate tracing of the surrounding collagen fibrils (area B). Perivascular fibrosis ratio (PFR) was calculated as area B over total perivascular area A + B (B/A + B).

Qualitative evaluation of ventricular interstitium

Interstitial fibrosis vs replacement fibrosis

Sections stained with hematoxylin-eosin and picosirius red (standard/polarized light; 10×/40×) were evaluated to identify one of two patterns of fibrosis. Interstitial fibrosis

was defined as increased interstitial and/or perivascular collagen without evidence of myocyte loss. Replacement fibrosis was defined as increased interstitial/ perivascular collagen with evidence of myocyte loss. Evidence for an inflammatory response, based on findings of myocardial leukocyte infiltration, was also sought.

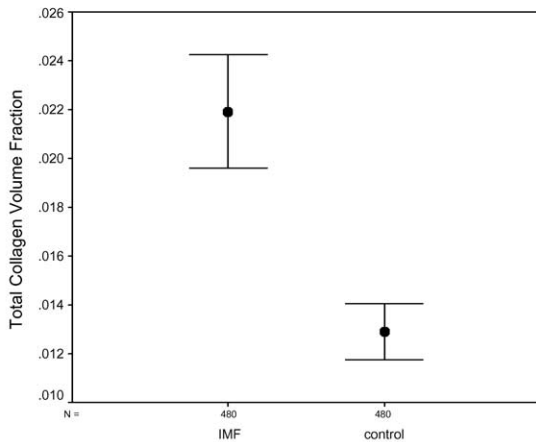
Apoptotic cell death

All sections were assessed for evidence of apoptotic cell death using the terminal transferase-mediated dUTP nick end-labeling (TUNEL) assay (ApopTag system, Serologicals Corporation, Temecula, CA, USA). ApopTag detection was validated using human tonsil²³ (standard light, 10× and 40×). Both case and control sections were used as negative controls, and phosphate-buffered saline substituted for deoxynucleotidyl transferase during the terminal reaction (standard light, 10× and 40×).

TGF-β₁ expression

Expression of TGF-β₁ was evaluated using an avidin-biotin-peroxidase technique as described previously.²⁴ Briefly, a mouse monoclonal antibody against human TGF-β₁ (1:200 dilution, clone TB21; Serotec Inc., Raleigh, NC, USA) was incubated with sections at 4°C overnight. Negative controls were incubated with phosphate-buffered saline under the same conditions. Subsequent incubation was conducted with a biotin-labeled, anti-mouse secondary antibody (1:20 dilution, room temperature, 30 minutes; Sigma-Aldrich, Inc., St. Louis, MO, USA) followed by incubation with an avidin-peroxidase conjugate (room temperature, 30 minutes). Each section was treated with diaminobezidine and counterstained with 0.5% methyl green. Staining intensity for TGF-β₁ was evaluated using a semi-quantitative grading system. Sections were viewed (standard light, 10× / 40×) and a score of 0 to 3 assigned

Panel A. Ventricular Collagen Volume Fraction



Panel B. Collagen Volume Fraction by Myocardial Region

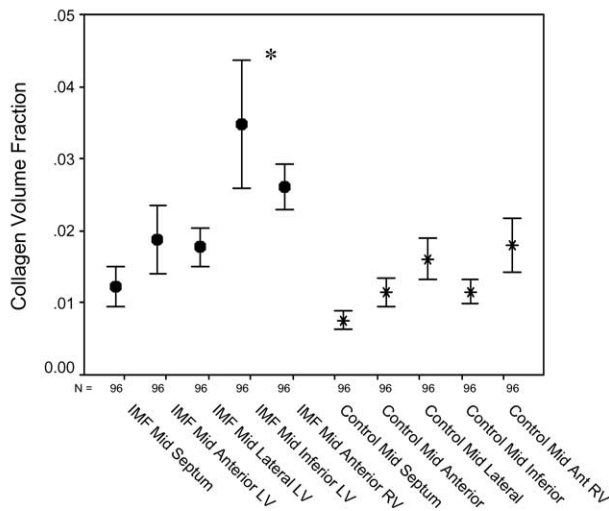


Figure 1 A. Comparisons of mean total CVF (95% CI) in IMF vs controls B. Heterogeneous increase in CVF in IMF vs controls. Mean CVF (95% CI) by myocardial region. N = number of digital fields analyzed. There is a wider range of CVF both within and between regions of myocardium in IMF. *CVF mid-inferior LV (IMF) was significantly greater than all other sections (ANOVA $p < .001$) except IMF mid-anterior RV.

based on the intensity of staining (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = intense staining). Perivascular, interstitial, and myocardial areas in each section were evaluated and scored separately. Total score for a section represented the average of the separately graded areas within each section.

Statistical analysis

All continuous data are reported as mean \pm standard error of mean. CVF and PFR were compared using independent samples t test (Level of significance $P < .05$). Bonferroni one-way analysis of variance was used to compare mean values across sections and Pearson's correlation

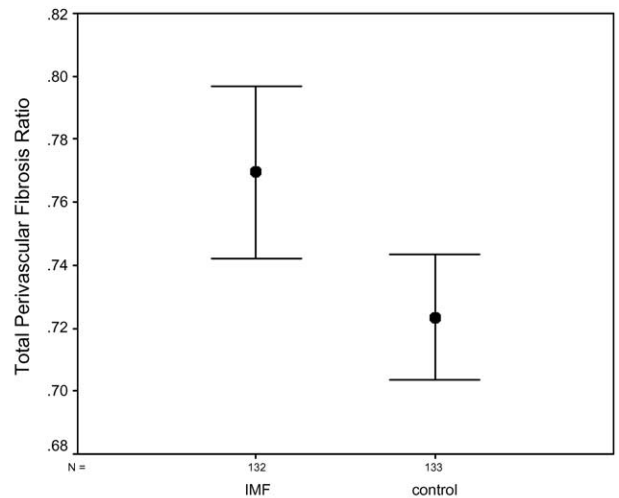
coefficients determined by pairing mean values for each section. TGF- β_1 staining intensity is expressed as mean \pm standard error of mean and compared using the Mann-Whitney U test. Spearman's rank correlation was used to assess correlation between CVF values and TGF- β_1 staining intensity.

Results

Clinical evaluation

Of the 270 consecutive cases of SCD, six patients had IMF (2.2%). Characteristics of IMF cases and controls are shown in Table 1. The sex distribution was four females and two males, matched in the control group. Mean age of IMF

Panel A. Overall Perivascular Fibrosis Ratio



Panel B. Perivascular Fibrosis Ratio by Myocardial Region

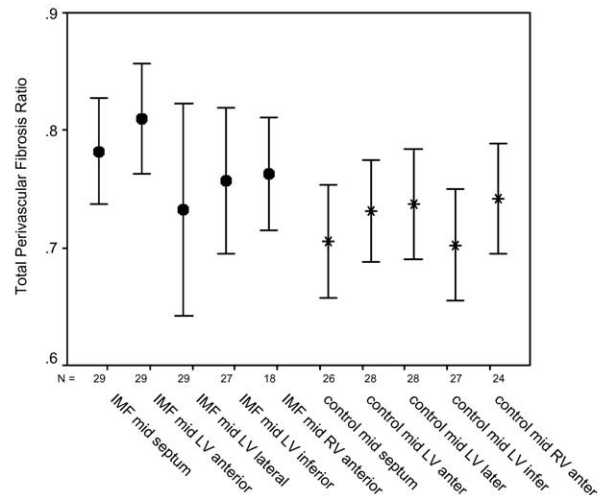


Figure 2 A. Mean total PFR (95% CI) in IMF vs controls B. Mean PFR (95% CI) by region of myocardium (n = number of arterioles measured).

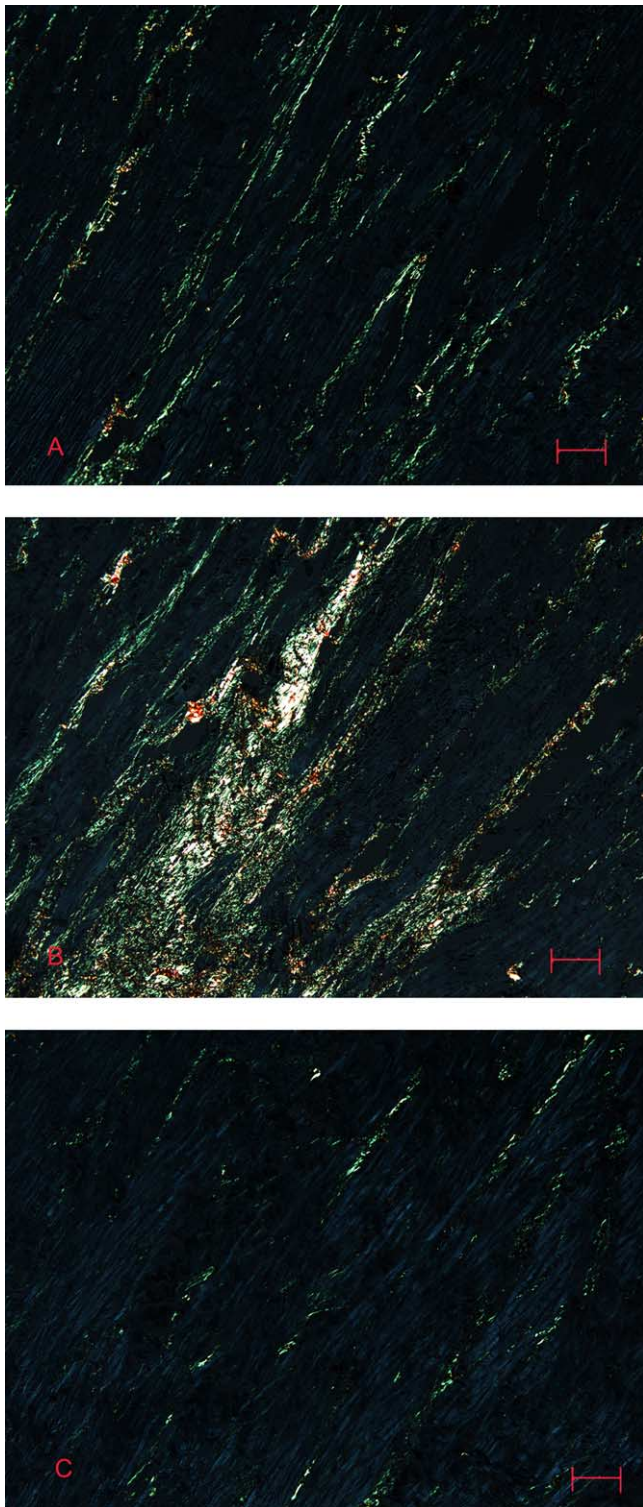


Figure 3 Representative photographs of picosirius red-stained myocardium ($10\times$ scale bar = $50\ \mu\text{m}$), showing two patterns of fibrosis **A**. Case #5, interstitial fibrosis with no evidence of myocyte loss **B**. Case #5, additional replacement fibrosis with evidence of myocyte loss **C**. Control subject #2.

cases was 33.7 ± 3.6 years and 34.3 ± 4.0 years for controls ($P = .9$). Average heart weight in IMF was 318.3 ± 20.8 g vs 284.2 ± 46.6 g in controls ($P = .52$). In all

subjects, there were no significant extracardiac findings on autopsy.

Collagen volume fraction

Overall CVF was significantly higher in IMF vs controls ($.022 \pm .001$ vs $.013 \pm .0001$, $P < .001$ (Table 2, Figure 1A). Increases in CVF were observed in multiple regions of the ventricular myocardium. The myocardial regions with the highest CVF were the LV inferior wall and RV mid-anterior wall (Figure 1B). CVF values were significantly more heterogeneous in IMF compared to controls, both within specific regions of myocardium as well as between regions (higher range of CVF, Figure 1B). There was no significant correlation between CVF and age ($r = -.113$, $P = .388$) or CVF and heart weight ($r = .144$, $P = .273$).

Perivascular fibrosis ratio

Perivascular fibrosis ratio (PFR) was also greater in cases vs controls (Table 2, Figure 2A) and this increase was observed in both ventricles. There were no significant differences in regional distribution of PFR between the two groups (Figure 2B). There was no significant association of PFR with age or heart weight ($r = .245$, $P = .063$ and $r = .006$, $P = .965$, respectively).

Pattern of fibrosis

In four IMF cases, the pattern of fibrosis was exclusively interstitial. In two patients, while the major contribution was from interstitial fibrosis, replacement fibrosis was also observed (cases 1 and 5). Control subjects showed no evidence of interstitial fibrosis or replacement fibrosis on gross and histological exam. Examples of both patterns in comparison with normal myocardium are shown in Figure 3.

Inflammation and apoptosis

There was no evidence of myocardial inflammatory response in either cases or controls. Also, evidence for apoptotic cell death was not observed in cases or controls.

TGF- β_1 expression

There was a consistently higher staining score for TGF- β_1 in cases vs controls, across all myocardial regions (Table 3). Overall score for cases was $2.36 \pm .06$ compared to $.99 \pm .06$ for the control group ($P < .001$). The myocardial region with the highest level of expression was the mid-inferior LV in IMF ($2.58 \pm .10$ vs $1.04 \pm .14$ in controls ($P < .001$). CVF correlated positively with TGF- β_1 intensity score ($r = .277$, $P = .03$). In IMF cases, TGF- β_1 intensity scores were high in the interstitial/ perivascular compartments as well as myocytes (Table 3). However, in

Table 3 Comparison of TGF- β_1 immunostaining intensity between IMF cases and controls

	IMF (n = 6)	Controls (n = 6)	P value*
TGF- β_1 intensity by location			
Perivascular	2.43 \pm 0.12	1.27 \pm 0.10	<.001
Interstitial	2.27 \pm 0.13	1.17 \pm 0.08	<.001
Myocardial	2.30 \pm 0.14	0.80 \pm 0.14	<.001
TGF- β_1 intensity by region			
Total	2.36 \pm 0.06	0.99 \pm 0.06	<.001
Left ventricle	2.44 \pm 0.06	0.99 \pm 0.07	<.001
Mid-septum LV	2.42 \pm 0.16	0.88 \pm 0.14	<.001
Mid-anterior LV	2.38 \pm 0.13	1.08 \pm 0.13	<.001
Mid-lateral LV	2.38 \pm 0.10	0.96 \pm 0.14	<.001
Mid-inferior LV	2.58 \pm 0.10	1.04 \pm 0.14	<.001
Mid-anterior RV	2.04 \pm 0.18	1.00 \pm 0.13	<.001

* Mann-Whitney *U* test.

controls, TGF- β_1 expression within myocytes was either weak or absent. TGF- β_1 expression in an IMF patient, a control subject, and a negative control are shown in Figure 4.

Discussion

In summary, there was evidence of diffuse and heterogeneous remodeling of the ventricular interstitium in IMF. We observed significantly higher myocardial CVF in 6 patients with IMF and SCD, compared to 6 age- and sex-matched controls. The greatest augmentation of collagen content was observed in the LV inferior wall. This was accompanied by significantly increased PFR in cases vs controls. The pattern of fibrosis in IMF was largely interstitial, but 2 patients had evidence of additional replacement fibrosis as well. Myocardial inflammation or apoptotic cell death were not observed. TGF- β_1 expression was significantly increased in IMF and correlated positively with myocardial CVF.

The postmortem observation of IMF in patients who suffer SCD is a relatively uncommon, but consistent, finding. Our observation of a 2.2% rate of occurrence among an adult SCD autopsy series over a 13-year period is similar to other autopsy series.⁷⁻⁹ However, available postmortem evaluations of such patients are limited, and detailed quantitative as well as qualitative studies are lacking. It is of interest that in previous reports, fibrosis in IMF patients was described as being mainly limited to the interventricular septum.²⁵⁻²⁷ However, in our series, idiopathic fibrosis was observed in multiple regions of the myocardium (Figure 1B). Since gross examination of these cases at autopsy had showed localized fibrosis, our observation of global alteration of the interstitium with diffusely increased CVF is an unexpected finding. Also, the distribution of myocardial collagen was significantly more heterogeneous in cases compared to controls. This was evident by the wider range of CVF values seen both within and between myocardial regions in the case group.

Mean CVF was highest in the LV inferior and RV mid-anterior walls; this was not observed in controls. Spach and co-workers have postulated that interstitial and perivascular collagen accumulation may interfere in side-to-side electrical coupling between myocytes, resulting in slow and discontinuous conduction.²⁸ It is conceivable that the diffuse but inhomogeneous increases in collagen observed in IMF could promote the genesis of reentrant ventricular arrhythmia by this mechanism. In this context, it is of interest that 3 of the case subjects had either documented arrhythmias or symptoms suggestive of arrhythmias prior to the occurrence of cardiac arrest.

The etiology and mechanisms of extensive interstitial remodeling among the IMF cases remain uncertain. Patients in our series were relatively young, which is likely related to Minnesota medical examiner practice directed by state regulations⁶ (overall autopsy series mean age 42 years; case group 34 years). Also, there was no correlation of CVF or PFR to age; thus these findings are unlikely to be related to the process of aging. By definition, patients were not included in the IMF group if heart weight was greater than 95% percentile based on body weight;¹⁹ thus the findings of fibrosis cannot be attributed to pathologic myocardial hypertrophy either. From a qualitative perspective, the case group mainly had an interstitial pattern of fibrosis, ie, increases in interstitial (CVF) and perivascular (PFR) connective tissue without destruction of myocytes. In animal models of myocarditis, interstitial fibrosis can be seen as a late development both with and without active inflammation.²⁹⁻³¹ Others have reported the occurrence of apoptotic cell death in patients with both acute and chronic forms of myocarditis.³² However, the postmortem diagnosis of myocarditis in humans requires the documentation of inflammatory infiltrates consisting mainly of lymphocytes, in addition to myocyte necrosis and significant replacement fibrosis.^{10,33,34} Since both inflammatory infiltrates and apoptosis were not observed, and replacement fibrosis was relatively uncommon among our cases, prior myocarditis is unlikely to have led to interstitial remodeling among IMF cases in the present study.

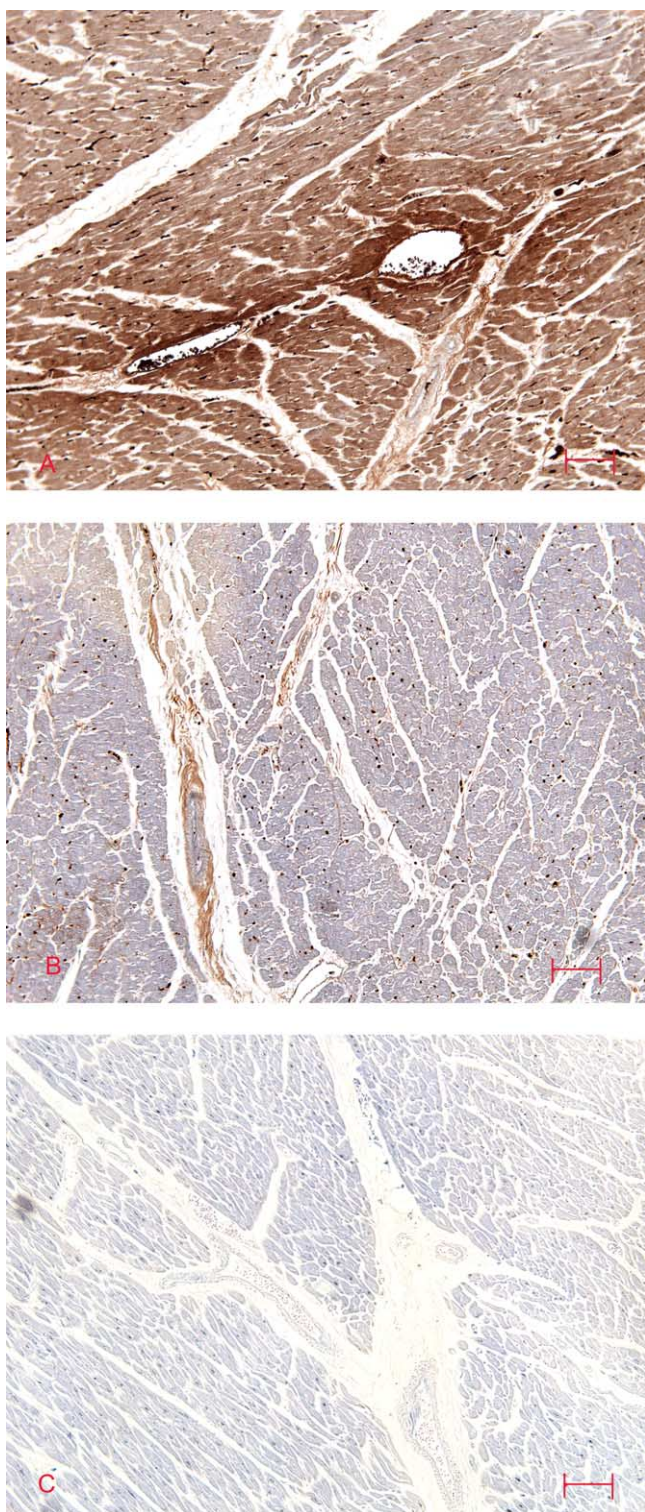


Figure 4 Representative photographs of TGF- β_1 immunostained myocardium (10 \times scale bar = 100 μ m) **A.** IMF case #2, intense staining seen in perivascular space and surrounding myocytes. **B.** Control # 2, mild staining seen in perivascular space, and no significant staining within myocytes. High-intensity staining within platelets due to platelet-derived TGF- β_1 is seen in both case and control. **C.** Case #5, negative control.

The diffuse nature of the process as well as low probability of previous myocarditis support the involvement of a humoral response in the pathogenesis of IMF. We observed significant overexpression of TGF- β_1 in IMF cases. TGF- β_1 is the prototype of the TGF- β superfamily of growth factors, which includes three isoforms of TGF- β , isoforms of bone morphogenic proteins, and activins.¹⁸ TGF- β_1 acts in an autocrine / paracrine fashion^{15,16} and promotes deposition of extracellular matrix both by increasing collagen synthesis and impairing collagen degradation.^{35–37} Some TGF- β_1 effects further occur via connective tissue growth factor (cell proliferation, adhesion, migration, and the synthesis of extracellular matrix) and this growth factor is also an important regulator of matrix metalloproteinase gene expression.^{18,38} In cardiac fibroblasts, the ability of angiotensin II to induce collagen synthesis may be mediated by increased TGF- β_1 production.³⁹ Overexpression of TGF- β_1 has been observed in several cardiac/other disease conditions as well as hypertension.^{40–43} In both animals and humans, acute and limited injury is accompanied by only a transient increase in TGF- β_1 , and fibrosis does not occur. The increase in TGF- β_1 production is sustained only with repeated injury, leading to the progressive deposition of extracellular matrix and tissue fibrosis.¹⁶ Among our cases, only one subject (case #6) had a clinical history of hypertension and none had cardiac hypertrophy by heart weight or wall thickness criteria. In the absence of a known injury or other explanation for the pathogenesis of IMF mediated by TGF- β_1 , one could speculate that occult hypertension (without LVH), novel gene defects, and/or novel circulating mediators may have been involved. Interestingly, in the present series of IMF, the most marked increase in TGF- β_1 expression was observed within myocytes, suggesting local myocardial production of this cytokine. The modest, but significant, correlation between TGF- β_1 expression and CVF suggests an association of this growth factor with myocardial interstitial fibrosis in IMF.

Limitations

The present study was retrospective in nature and the fundamental nature of postmortem evaluations limits their scope to association; causal inferences should be made with due caution. However, since there were no extracardiac findings at autopsy, in any of the subjects, noncardiac causes of sudden death would be highly unlikely. While the presenting arrhythmia at time of cardiac arrest was not available, all patients either had a witnessed cardiac arrest or arrested within one hour of initial symptoms. Further elucidation of the etiology and pathogenesis of IMF will require large prospective studies with access to clinical information, myocardial tissue, and serum of subjects who suffer SCD.

Conclusions

Idiopathic myocardial fibrosis associated with sudden cardiac death is a process that involves diffuse and heteroge-

neous remodeling of the ventricular interstitium, with the greatest degree of collagen augmentation observed in the LV inferior wall. While TGF- β_1 is a potential mediator, elucidation of the initial inciting injury and/or predisposing factors awaits further investigation. Our findings underscore the utility of comprehensive histopathologic examination for the evaluation of a complex phenotype such as sudden cardiac death.

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