Fibrosis in the Transplanted Heart and Its Relation to Donor Ischemic Time
Assessment With Polarized Light Microscopy and Digital Image Analysis

J. Geoffrey Pickering, MD, FRCPC, and Derek R. Boughner, MD, PhD, FRCPC

Long-distance procurement of cardiac allografts is commonly used to increase the supply of donor organs but has recently been associated with the development of impaired diastolic function. Therefore, the effect of the post-ischemic duration on myocardial fibrosis was quantitatively evaluated in 36 cardiac transplant recipients in whom the ischemic time ranged from 70 to 363 (mean, 189±83) minutes. Interstitial collagen was quantified with polarization microscopy and digital image analysis in 115 endomyocardial biopsy specimens taken 5–10 days after surgery. The technique, developed for this study, showed excellent correlation with hydroxyproline analysis (r=0.98, p<0.001). Collagen volume fraction in biopsy specimens from the transplanted hearts was significantly greater than that in biopsy samples from seven normal, age-matched autopsy hearts (4.7±1.9% vs. 2.9±0.6%, p<0.02). The degree of fibrosis correlated with the total ischemic time (r=0.60, p<0.001). Donor age ranged from 10 to 71 years and did not correlate with the degree of fibrosis. No relation was found between the corresponding collagen content and right atrial pressure, pulmonary artery wedge pressure, or cardiac output measured at the time of biopsy. Myocyte damage was observed in eight of the 36 patients and was characterized by a striking loss of muscle birefringence. We conclude that cardiac allograft fibrosis may be identified shortly after transplantation and is dependent on the total ischemic duration. (Circulation 1990;81:949–958)

Cardiac transplantation has become an important therapeutic alternative for patients with end-stage heart disease. Although exercise performance is relatively good after transplantation,1,2 several studies have reported impaired diastolic function of the transplanted heart.3–7 Recently, increased myocardial stiffness was demonstrated after 1 year and was found to be related to the ischemic time of the donor heart.8 This suggests that myocardial scarring may be a functionally important sequela of prolonged ischemic times. Yet, with the dramatic increase in frequency of cardiac transplantation,9 reliance on off-site, long-distance organ procurements is expected to increase.10,11

Therefore, it would be useful to establish the nature of the relation between myocardial fibrosis and the ischemic time of the donor heart. Present methods for quantifying fibrosis in endomyocardial biopsy samples, however, are limited either by unacceptable interobserver variability12 or impracticality (e.g., hydroxyproline measurement or stereologic techniques). To reliably evaluate fibrosis in endomyocardial biopsy samples, we developed a videodensitometry system that exploits the birefringent properties of fibrillar collagen stained with picrosirius red.13 We report here the accuracy of this technique in comparison with hydroxyproline analysis. Furthermore, the methodology was used to investigate the relation between cardiac fibrosis and ischemic time in a consecutive series of heart transplant recipients.

Methods

From a consecutive series of 40 orthotopic heart transplant recipients, 36 patients in whom at least three myocardial biopsy samples were available for analysis were studied. The mean donor age was 26±11 years.

Donor Heart Retrieval

Graft excision was performed based on the technique of Shumway et al.14 Twenty-six (72%) of the donors required inotropic support before graft har-
vesting. The cardioplegia solution consisted of Ringer's lactate with 20 mg/l dexamethasone, 3,200 units/l heparin, 25 g/l dextrose, 10 units/l regular insulin and was supplemented with 3.6 mmol/l sodium bicarbonate and 20 mmol/l KCl. The donor hearts were wrapped in sterile towels, placed in a plastic bag, and immersed in an iced saline solution. During long-distance transport, temperature was continuously monitored and maintained between 4° and 6°C. The total ischemic time was defined as the time from clamping of the donor aorta during harvesting to unclamping of the recipient aorta after aortic anastomosis. For the group, this ranged from 70 to 363 minutes and from 0 to 120 minutes in 12 cases, 121 to 240 minutes in 14 cases, and more than 240 minutes in 10 cases.

Digital Imaging Methods

The videodensitometry system was designed around an Optiphot-Pol polarizing light microscope (Nikon). Light was transmitted through a ×10 objective and a ×1 TV relay lens to a solid-state CCD video camera (series 4810, Cohu, San Diego, California) mounted on the eyepiece tube. Resolution of the camera was 754 (horizontal)×488 (vertical) photosites. Images were digitized by a videoframe grabber (PCVISION plus, Imaging Technology, Woburn, Massachusetts) at a resolution of 640 (horizontal)×480 (vertical) pixels with eight-bit pixels (256 gray levels). Pixel size was 1.82 μm². Digitized images were displayed on a high-resolution monitor (model C-3479, Mitsubishi, Torrance, California). A linear light response was confirmed with a series of progressively exposed radiographic films mounted on glass slides, and the respective optical densities were measured by a densitometer (model 301, Xrite, Grand Rapids, Michigan).

When stained with picrosirius red, collagen is readily identified with polarization microscopy as bright yellow-orange or green structures (Figure 1A). To optimize the contrast in brightness between collagen and cardiac muscle, which is the other birefringent component of myocardium, specimens were stained with picrosirius red and illuminated with polarized light of varying wavelengths controlled by a monochromator (Bausch and Lomb). In this way, collagen was found to become optimally distinct from muscle (brighter) when illuminated with light of 600-nm wavelength. Therefore, a 600±5-nm band-pass filter (Optikon, Waterloo, Canada) was used for all collagen determinations. The black-level setting of the camera was manually increased by 11% to eliminate any light response to cardiac muscle. Thus, the final digitized image consisted of two components: noncollagenous material appearing black (gray-level 0) and collagen appearing in various degrees of brightness (gray-levels 1–255) (Figures 1B and 2A). The total fibrous content could then be expressed as the area fraction of bright pixels.

To quantify the total area of myocardium being analyzed, we illuminated the picrosirius red–stained sections with nonpolarized light through a 540±5-nm band-pass filter (Optikon). In this way, the nontissue background, including artifactual gaps in the sample, were readily distinguishable from the remainder of the sample, again based on gray levels (Figure 2B). An identical intensity of illumination was used for all measurements.

Validation of collagen quantification by videodensitometry. Hydroxyproline analysis served as the standard of comparison for this study, although its value in the routine assessment of transplanted hearts is limited because it would require the destruction of at least one and possibly several biopsy samples. Fourteen autopsy hearts fixed in 10% neutral-buffered formalin were selected for study. Five were from patients with no clinical evidence of heart disease, and the others were from patients with a history of myocardial infarction (six patients) or dilated cardiomyopathy (three patients). Endomyocardial samples were excised from the interventricular septum and were divided perpendicular to the endocardial surface into two pieces. The endocardial itself was dissected free of the specimen to avoid the possibility of unequal division of endocardial collagen. One of the two portions was then embedded in paraffin, and the cut surface was sectioned at a 5-μm thickness. All 14 specimens were simultaneously stained with picrosirius red, and the entire section was analyzed for collagen content by videodensitometry. The second portion of each sample was dried at 60°C to a constant weight, hydrolyzed in 6N hydrochloric acid at 105°C, and dried under vacuum. Hydroxyproline analysis was performed according to Chiariello et al. Briefly, specimens were oxidized with a chloramine-T solution followed by addition of Erlich's reagent in perchloric acid. Absorbance was measured at 558 nm, and results were expressed as nanomoles of hydroxyproline per milligram of dry weight. The two quantification approaches were performed independently by investigators unaware of the results of the alternate approach.

Analysis of Biopsy Samples and of Hemodynamic Data From Transplanted Hearts

Right heart hemodynamics were measured with a Swan-Ganz thermodilution catheter 1 week after heart transplantation for the purpose of this and other ongoing studies. On the same occasion, multiple right ventricular endomyocardial biopsy samples were obtained with a Stanford-Caves bioprobe. This set of biopsies was chosen for study because they were the initial biopsy specimens obtained from all patients, thereby eliminating the possibility of scarring from previous biopsy procedures and minimizing the likelihood of rejection-related fibrosis. Biopsy samples were immediately fixed in 10% neutral buffered formalin, embedded as a single paraffin block, and sectioned at a 5-μm thickness. Following the routine procedure at this center, we had the slides stained with hematoxylin and eosin, methyl-green pyronine, and Masson's trichrome. A diagnosis of
FIGURE 1. Panel A: Photomicrograph of a section of interventricular septum stained with picrosirius red and viewed through the polarized light microscope. Collagen appears either bright yellow-orange or bright green, whereas muscle is faintly visible and appears dull green. Bar, 75 μm. Panel B: Digitized image of the same section as in Panel A. Image thresholding has been applied so that all gray levels above 0 (black) are set to 255 (maximum brightness), illustrating the two-component nature of the image. Bar, 75 μm.

rejection was made according to the criteria of Billingham. For the purpose of this study, a slide adjacent to the hematoxylin and eosin–stained slide was stained with picrosirius red. This was done in two batches with concomitant staining of a section of rat tail tendon serving as a control tissue. When illuminated with polarized light, the median gray levels of the digitized image of the tendons were identical, and staining conditions were inferred to be equal for both sets of biopsy samples.

The collagen content for each biopsy sample was determined by videodensitometry without knowledge of the corresponding ischemic time. The endocardium was carefully excluded from the analyzed field.
as were blood vessels and perivascular tissue. This was facilitated by use of a mouse-controlled system for including or excluding areas of interest. To give proportional weight to biopsy specimens containing relatively more myocardium, the collagen estimate for each patient was expressed as the total collagen content for all biopsy samples divided by the total area of myocardium analyzed.

Histologic evidence for ischemic myocyte damage was sought based on focal myocytolysis in the absence of lymphocytic infiltration with or without contraction bands. To assist in the early recognition of ischemic changes, the sections stained with hematoxylin and eosin were examined under polarized light for a loss of muscle birefringence (visibility) and for contraction bands that appear bright under these conditions. The presence of contraction bands alone was not considered sufficient to diagnose ischemic damage.17

Collagen Analysis of Biopsy Samples From Normal Hearts

Seven autopsy hearts from patients free of cardiac disease were studied. The mean age of the patients at the time of death was 28±7 years. The cause of death was motor vehicle accident in three patients, drug overdose in two, chronic active hepatitis in one, and lymphoma in one. With a similar biotome to that used in the heart transplant recipients, three or four endomyocardial biopsy samples from the right interventricular septum were taken from each heart, sectioned, and stained with picrosirius red. Staining conditions comparable to those of the transplant heart biopsy samples were verified by the rat tail
tendon control tissue. The collagen estimate for each biopsy sample was made by videodensitometry.

Statistical Analysis

Values are given as mean±SD. Ischemic times were analyzed in three categories: 0–120, 121–240, and more than 240 minutes. Comparisons between groups were performed with one-way analysis of variance and Newman-Keul’s multiple-range test. Noncontinuous variables were evaluated by contingency table analysis. Relations between the two methods for quantifying collagen, collagen content and ischemic time, and hemodynamics and collagen content were tested by linear regression analysis.

Results

Validation of Videodensitometry Technique

A wide range in the values of hydroxyproline content existed in the myocardial samples from the 14 autopsy hearts used in the validation study (19.2–268.8 nmol/mg dry wt). The histologic features included normal myocardium, varying degrees of interstitial fibrosis, and areas with dense scar tissue. The collagen volume fraction, determined by videodensitometry, ranged from 3.7% to 50.4%. As depicted in Figure 3, there was good correlation between the hydroxyproline content and the collagen estimate by videodensitometry (r=0.98, p<0.001).

Analysis of Biopsy Samples From Normal and Transplanted Hearts

From the seven normal autopsy hearts, a total of 23 biopsy samples were taken (3.3±0.5 biopsies/heart). The area of myocardium analyzed for each biopsy was 1.9±0.7 mm². The myocardial collagen content was 2.9±0.6%. From the 36 transplant recipients, biopsy samples were obtained 7.7±1.6 (range, 5–10) days after surgery. One hundred fifteen biopsy specimens were evaluated (3.2±0.4 biopsy samples/patient), and the average area of myocardium analyzed for each biopsy sample was 1.2±0.6 mm². The mean collagen content was 4.7±1.9% (range, 1.5–9.9%) and was significantly greater than that of the control hearts (p=0.02). Increased collagen typically appeared as focal patches of fibrosis that were best visualized with polarization microscopy (Figure 4).

Total allograft ischemic time was 0–120 minutes in 12 patients, 121–240 minutes in 14, and more than 240 in 10. The age of the hearts and the proportion of donors requiring inotropic support were not different between groups (Table 1). Biopsy samples from hearts with the longest ischemic times (>240 minutes) had significantly more myocardial collagen than those in the other groups (p<0.05). Biopsy samples from hearts with an intermediate ischemic time (121–240 minutes) were more fibrotic than those from normal autopsy hearts (p<0.05). Regression analysis revealed a linear correlation between myocardial collagen content and ischemic time (r=0.60, p<0.001) (Figure 5). There was no association between myocardial collagen content and the donor age (r=−0.41).

The mean right atrial pressure, pulmonary artery wedge pressure, and cardiac output were 9.0±5.0 mm Hg, 14.9±4.7 mm Hg, and 5.5±1.0 l/min, respectively. Neither the pulmonary wedge pressure nor the cardiac output correlated with the collagen content (r=0.01, r=0.04, respectively). A trend was observed between the right atrial pressure and the collagen content, although this trend was not significant (r=0.18, p=0.07).

Myocyte necrosis was seen in biopsy samples from eight patients. The mean ischemic time for this group was 244±66 minutes, and the collagen content was 5.7±1.5%. Damaged myocytes characteristically revealed reduced or absent birefringence (Figure 6). Bright contraction bands were readily apparent at the periphery of the necrotic regions (Figure 6), although they were also commonly seen in biopsy samples without myocytolysis. In one patient, a lymphocytic infiltrate with pyroninophilia was associated with the myocytolysis. This was the only patient in whom rejection was identified.

Discussion

Diastolic abnormalities have been documented in the cardiac transplant recipient and include elevated right atrial and pulmonary wedge pressures, increased left ventricular end-diastolic pressures, and abnormal right atrial pressure waveforms. Although ventricular relaxation appears to be normal, the passive stiffness of the ventricle has been shown to be increased. This is consistent with a significant increase in the fibrous component of the ventricle. In turn, the relation between conditions predisposing to scarring and the actual severity of myocardial fibrosis should be delineated. We chose to specifically examine the effect of the allograft ischemic time on fibrosis for two reasons: first, because of the pressure to use long-distance procurements and, second, because the ischemic time is the only parameter to date that has been positively correlated with the passive diastolic properties of the ventricle.
A prerequisite for correlating histologic features with clinical parameters is the use of an objective approach that yields quantitative morphological data. Such an approach was recently used for the assessment of myocyte hypertrophy in cardiac recipients.\textsuperscript{20} The present study is the first to quantify myocardial fibrosis in heart transplant recipients.

**Technique of Collagen Quantification**

Polarization microscopy with picrosirius red was first used by Constantine and Mowry\textsuperscript{21} to study dermal collagen. More recently, it was used to measure the molecular and fiber orientation of collagen in myocardial infarct scars\textsuperscript{22} and to characterize collagen fiber arrangement in experimentally hypertrophied and normal hearts\textsuperscript{23} and in human hearts with dilated cardiomyopathy.\textsuperscript{24} We have found that the technique is ideally suited for collagen videodensitometry because it offers an approach based purely on intensity of brightness. Morphometry techniques based primarily on differences in color frequently suffer from imperfect segmentation of features and the need for image editing.\textsuperscript{25} For a given section thickness, the birefringence of collagen is approximately seven times that of muscle; therefore, the two are readily distinguishable with polarized light tech-

\begin{table}
\centering
\caption{Characteristics of Normal and Transplanted Hearts}
\begin{tabular}{l l l l l}
\hline
 & & & & \\
 & Normal heart & & & \\
& \textit{(n=7)} & Ischemic time (min) & 0–120 & 121–240 & >240 \\
\hline
Ischemic time (min) & \ldots & 96±15 & 193±29 & 295±36 \\
Age (yr) & 28±7 & 32±14 & 23±9 & 22±5 \\
Patients on inotropes & \ldots & 9 & 11 & 7 \\
Collagen content (\%) & 2.9±0.6 & 3.4±1.3 & 4.4±1.2* & 6.7±1.7† \\
Patients with myocyte necrosis & \ldots & 1 & 3 & 4 \\
\hline
\end{tabular}
\end{table}

*\textit{p}<0.05 vs. normal, \textit{f}p<0.05 vs. normal, 0–120, 121–240.
FIGURE 5. Relation between total allograft ischemic time and myocardial collagen content of biopsy samples taken 5–10 days after transplantation. One patient with an ischemic time of 225 minutes and a collagen content of 6.3% also had acute rejection.

niques. All other cardiac components, being nonbirefringent, are invisible when viewed with the polarizing microscope.

After validating the technique, we used it to assess the collagen content of biopsy samples from normal autopsy hearts, which was found to be approximately 3%. This is similar to the results of a morphometric study by Pearman et al20 of five normal autopsy hearts that revealed a collagen volume fraction between 2% and 3%.

Collagen Content After Transplantation and Relation to Procurement Time

The dramatic increase in the worldwide frequency of cardiac transplantation9 has placed increased demands on the limited supply of donor organs. To offset this problem, long-distance procurement, with the attendant period of ischemia, has become increasingly used.10 However this approach to expanding the pool of available organs must be balanced against the risks of ischemic injury, which are clearly time dependent.27Billingham et al11 have shown that with ischemic times less than 3 hours capillary endothelial changes are prevalent after reperfusion and are worse in long-distance procurements.11 In the immediate days after transplantation, myocyte damage, presumably ischemic, is a biopsy finding common to several transplant centers.10,28,29 It is expected that any irreversible myocyte damage would progress to focal myocyte fibrosis within several days.30

By quantifying myocardial collagen in the initial biopsy samples of heart transplant recipients, we found that by 5–10 days the fibrous content was already slightly greater than normal. Furthermore, a relation existed between the collagen content and the total ischemic duration suggesting that the ischemic period is indeed an important etiology for allograft fibrosis and should be considered when scarring is identified in the weeks after surgery. Excess collagen was typically seen as fibrotic patches with abundant fibroblasts. Frequently, but not always, focal myocyte necrosis was associated with the patches.

The 1-week biopsy sample was chosen for analysis to minimize interpretation difficulties arising from collagen deposition due to nonischemic etiologies. Myocardial fibrosis has been frequently observed in biopsy samples and may be related to resolved or resolving acute rejection,16,28,31 chronic rejection,32 cyclosporin,16,33 infection, and scarring from previous biopsies.28,31 Thus, to specifically identify ischemia-induced fibrosis, an early assessment is required. This may not be maximally sensitive because not all necrotic lesions may be at the fibrotic stage; however, in the group studied here, the mean time to performing the first biopsy was approximately 8 days, which is somewhat later than in other reports.6,28 With time, the relation between ischemic time and ischemia-induced fibrosis may be expected to strengthen, although this would be difficult to determine for the above reasons.

A potential limitation in interpreting data from endomyocardial biopsy samples is the degree to which the samples reflect the morphological structure of the remainder of the myocardium. In the present study, patients were included if at least three satisfactory biopsy specimens were available for analysis, although it has been suggested that five specimens provide a more reliable sampling of the myocardium.34

Assessment of Ischemia-Related Myocyte Changes

Polarization microscopy offers a unique approach to the assessment of cardiac muscle damage. As myofibrils become structurally disorganized, their optical properties are altered, resulting in a loss of myocyte birefringence.35 This becomes manifest as reduced muscle visibility when the microscope analyzer is inserted into the light path. Although a mild reduction in birefringence may be reversible, a complete absence suggests extensive fibrillar and molecular degradation that is consistent with cell death. At times, we observed complete loss of visibility when only minor changes were evident on routine microscopy of the hematoxylin and eosin– and trichrome-stained sections. Thus, the approach may be particularly useful in distinguishing reversible from irreversible myocyte damage shortly after ischemia.

In total, myocyte necrosis was present in eight (25%) patients and tended to be more prevalent in patients with ischemic times between 4 and 6 hours, although this was not statistically significant, perhaps because of the relatively low frequency of the observation.

The enhanced birefringence of contraction bands is a polarized microscopy finding not previously described but clearly facilitates identification of this feature. It cannot be said for certain whether contraction bands represent early ischemic changes or an artifact of the biopsy procedure, but the increased birefringence is consistent with excessive interdigita-
tion of myofilaments in the absence of muscle protein disruption.

Functional Significance of Ischemia-Associated Fibrosis

The study did not reveal a relation between fibrosis and hemodynamics 5–10 days after transplantation. This lack of correlation may, in part, be due to the relatively mild degree of fibrosis seen at this time. In addition, measurement of diastolic pressures alone is likely too insensitive to reliably detect the functional effects of early myocardial fibrosis. Rather, indexes of the viscoelastic properties of the ventricle will more closely correlate with the degree of fibrosis. In this regard, Hausdorf et al. found increased myocardial stiffness in patients later than 1 year after surgery. Furthermore, they observed a correlation between ischemic time and the degree of both myocardial and chamber stiffness. The present study therefore supports the hypothesis that prolonged ischemic times, by predisposing to myocardial fibrosis, contributes to diastolic dysfunction in transplant recipients.

Elevated left ventricular end-diastolic pressure and atypical right atrial pressure responses to volume loading and inspiration have sporadically been observed. The inconsistency of such observations may
Conclusions

In summary, the technique of polarization microscopy of myocardium stained with picrosirius red has provided the basis for an accurate videoedensitometric approach to the quantification of myocardial fibrosis in the transplant recipient. Also, a novel assessment of myocyte injury after ischemia can be made, based on the immediate loss of myocyte structural integrity. With these approaches, we have shown that ischemia-associated allograft fibrosis may be identified shortly after transplantation and that its severity is dependent on the procurement time. Because fibrosis may contribute to impaired diastolic function, this relation should be considered when evaluating donor heart acceptability.

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J G Pickering and D R Boughner

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