Different microcirculatory and interstitial matrix patterns in idiopathic dilated cardiomyopathy and Chagas’ disease: a three dimensional confocal microscopy study

M de Lourdes Higuchi, S Fukasawa, T De Brito, L C Parzianello, G Bellotti, J A F Ramires

Abstract

Objective—To analyse the morphological aspects of the extracellular matrix and microcirculation to clarify whether chronic Chagas’ cardiopathy (CCC) is an accurate model to study the pathogenesis of idiopathic dilated cardiomyopathy (IDCM).

Design—Thick histological myocardial sections were prepared to analyse collagen, and microcirculation was examined during confocal laser and light microscopy.

Setting—The specimens were prepared at the pathology service of the Heart Institute of São Paulo, Brazil.

Patients—Nine control hearts, eight IDCM hearts, and 10 CCC hearts were studied after necropsy.

Main outcome measures—The number of collagen struts per 100× field, the area of fibrosis (%), and the diameters of arterioles and capillaries were measured in each heart to establish outcome.

Results—A smaller number (mean (SD)) of collagen struts was seen in the hearts in the IDCM group (9.1 (4.1)) than in the control (22.4 (3.2)) (p < 0.05) or CCC (15.7 (7.4)) (p > 0.05) groups. Fibrosis was greater in the CCC hearts (13.8 (10.5)%) than in the IDCM hearts (5.9 (6.6)% (p > 0.05). Major increases in arteriole (65.4 (9.9) μm) and capillary (9.9 (1.7) μm) diameters were seen in the CCC hearts but not in the IDCM hearts (arteriole diameter 40.3 (7.9) μm; capillary diameter 7.9 (1.3) μm).

Conclusions—Hearts demonstrating CCC and IDCM present different extracellular and microvessel alterations. This suggests that distinct pathogenic mechanisms are responsible for each condition and that CCC is not an effective model to study IDCM.

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Keywords: microcirculation; Chagas’ disease; dilated cardiomyopathy; extracellular matrix

Chronic Chagas’ cardiopathy (CCC) is regarded as an example of dilated cardiomyopathy of definite aetiology. Therefore, CCC is considered to be a useful model to investigate the pathogenesis of chronic heart failure caused by dilated cardiomyopathy.1–3

Microinfarcts, myocytolysis, hyaline degeneration, and fibrosis are common findings in CCC and have been attributed, in varying degrees, to chronic myocarditis, immunological phenomena, and microvascular alterations.4

The presence of arteritis,5–7 autonomic denervation,8 microspasm,9 microthrombosis,10 or vessel disarray11 apparently constitutes the vascular alterations responsible for ischaemic lesions; however, definite proof of major vascular damage in CCC is still lacking.12 13

In human idiopathic dilated cardiomyopathy (IDCM), an increase in total myocardial collagen, mainly type I collagen, has been shown.14 15 In this setting, coronary flow seems severely impaired,16 suggesting that fibrosis may result from myocardial ischemia.

There are no previous morphological studies comparing the extracellular matrix and microcirculation in human CCC and IDCM hearts.

Such morphological data may help clarify whether CCC is indeed an appropriate model for IDCM.

Materials and methods

A three dimensional histopathological analysis was performed in thick myocardial sections from normal, IDCM, and CCC hearts. All methods were approved by the institution’s ethics committee.

STUDY GROUPS

Three groups of hearts, obtained from necropsies and heart transplant recipients, were studied. Group A included normal hearts; group B, hearts from patients with IDCM; and group C, hearts from CCC patients with heart failure. All patients in groups B and C were classified as having New York Heart Association class III or IV heart failure and received varying doses of digoxin, frusemide, and captopril. The clinical data of these subjects are shown in table 1.

EXTRACELLULAR MATRIX STUDY

We analysed four hearts from group A, five hearts from group B, and five hearts from group C. The hearts were fixed in 10% formalin, and three consecutive transmural fragments, 20 mm in length from the apex to lateral wall of the left ventricle of each heart, were stained using the modified Hortega method.17 Adjacent fragments were sampled and routinely processed for paraffin embedded
blocks; 20 µm histological sections were stained with sirius red and studied using the confocal laser microscope Odyssey XL (Noran Instruments, Middleton, Wisconsin, USA).

**QUANTIFICATION OF STRUTS**

For each fragment, a 40 µm thick, formalin fixed section was refixed in bromide formalin for 24 hours, heated to 45°C, and submitted to the Hortega technique. The remains of the fragment were processed routinely, embedded in paraffin, sectioned at 3 µm thickness, and stained with Masson trichrome.

The modified Hortega method results in silver impregnation of the thin, fibrillar architectural collagen (supposedly type III collagen) and pink stained, thick, dense, interstitial, and reparative collagen fibres (probably type I collagen).

Thin, lateral connective fibres interconnecting myocytes are named struts. In this study, we defined struts as all connective tissue fibres crossing at least two myocardial fibres. Initially, we analysed 300 successive microscopic fields at 100× magnification, from the endocardium to the epicardium, in a normal myocardial fragment. A Student’s t test showed that 10 fields of medium magnification (field of 175.6±3 µm²), regularly distributed throughout the transmural myocardial wall, were representative of the fragment. A similar study was also performed in IDCM cases and showed the same results. We counted 30 fields, at 100× magnification, from each heart.

**QUANTIFICATION OF FIBROSIS**

The areas of fibrosis were determined by the Masson trichrome stain (in blue), in 30 fields, at 100× magnification at the third medium of each of the myocardial sections to avoid inaccurate comparisons caused by the differences between fibrosis present in the subendocardial and subepicardial regions. Quantification was performed with an Image processing and analysis system (Quantimet 500+, Leica Cambridge, Cambridge, UK).

**MICROCIRCULATION STUDY**

The three dimensional study of microcirculation was performed according to a modification of the technique described by Fujimoto and colleagues. Fresh hearts taken at necropsy were perfused with various solutions at a constant pressure (120 mm Hg). The “coronaria ostia” were cannulated, and the circulatory vessels were flushed with 300 ml of phosphate buffered saline containing luke-warm 5% glucose. A mixture of 2.5% glutaraldehyde and 2.0% paraformaldehyde in phosphate buffered saline was perfused for seven minutes, followed by perfusion of 0.5% silver nitrate in 5% aqueous glucose solution for five minutes, to impregnate the endothelial surface of the epicardial arteries, intramural arterioles, and capillaries. The fixative solution was reperfused for 10 minutes. The fixation of the heart was completed by total immersion in 10% formalin for 24 hours.

Six transmural fragments, each 20 mm in length, from the left ventricle of each heart were sampled. Three fragments were from the apex and adjacent lateral wall, and another three fragments from the septum, and medium portion of the lateral and anterior walls). The 40 µm thick cryostat sections, embedded in glycerol, were analysed as a three dimensional image using the reflected laser channel of the confocal microscope. Adjacent myocardial fragments were sampled for routine haematoxylin
and eosin staining. We analysed five hearts from subjects in group A, three hearts in group B, and five hearts in group C. The diameters of 10 arterioles, the penultimate ones just before capillary formation, from different areas of each heart, and 15 capillaries were measured in two-dimensional sections selected from serially acquired images in the z plane.

STATISTICAL ANALYSIS
The Kruskal-Wallis test was applied to establish whether the mean number of struts, mean area of fibrosis, and mean arteriole and capillary diameters differed among the groups with normal hearts, CCC hearts, and IDCMM hearts.

RESULTS
EXTRACELLULAR MATRIX STUDY
The main clinical data and quantitative analyses of fibrosis and struts are shown in table 1.

Normal hearts (group A)
The mean (SD) age of the patients was 30.7 (20.4) years and the mean weight of the hearts was 257.5 (29.9) g. Hearts were taken at necropsy from three patients who had died of haematological diseases. All three deaths lacked involvement of the heart. One of these normal hearts came from a potential heart donor; the heart was eventually deemed unsuitable for organ donation.

The mean number of transverse fibrillar collagen fibres (struts) per 200× microscopic field...
Table 2  Clinical data and pathological findings of arteriole and capillary diameters in patients with normal, IDCM, and CCC hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>Heart weight (g)</th>
<th>Arteriole diameter (µm)</th>
<th>Capillary diameter (µm)</th>
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<tr>
<td>Group A</td>
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<td></td>
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<tr>
<td>(normal hearts)</td>
<td>53</td>
<td>M</td>
<td>280</td>
<td>30.7</td>
<td>7.8</td>
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<tr>
<td></td>
<td>38</td>
<td>M</td>
<td>320</td>
<td>22.5</td>
<td>7.2</td>
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<tr>
<td></td>
<td>42</td>
<td>F</td>
<td>240</td>
<td>33.6</td>
<td>8.6</td>
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<tr>
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<td>49</td>
<td>F</td>
<td>260</td>
<td>23.7</td>
<td>7.7</td>
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<tr>
<td></td>
<td>46</td>
<td>M</td>
<td>330</td>
<td>33.5</td>
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<td>SD</td>
<td>5.3</td>
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<td>Group B</td>
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<td>(IDCM hearts)</td>
<td>73</td>
<td>M</td>
<td>560</td>
<td>40.8</td>
<td>9.4</td>
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<td>47</td>
<td>M</td>
<td>500</td>
<td>32.2</td>
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<tr>
<td></td>
<td>45</td>
<td>M</td>
<td>550</td>
<td>48.5</td>
<td>7.5</td>
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<td></td>
<td></td>
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<td>Mean</td>
<td>40.3</td>
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<td></td>
<td>SD</td>
<td>7.9</td>
<td>1.3</td>
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<td>Group C</td>
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<tr>
<td>(CCC hearts)</td>
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<td>M</td>
<td>350</td>
<td>83.5</td>
<td>10.5</td>
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<tr>
<td></td>
<td>48</td>
<td>M</td>
<td>600</td>
<td>50.1</td>
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<td>Mean</td>
<td>65.4</td>
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<td>SD</td>
<td>13.9</td>
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The mean age of patients in group C was 42.2 (14.3) years and the mean heart weight was 468.0 (62.61) g. The mean number of lateral struts was 15.7 (7.4) and varied considerably. Two hearts exhibited normal mean values and three had lower values (table 1). The remaining struts were frequently thickened (fig 1C). No significant difference was found when compared with normal hearts. The area of fibrosis was 13.8 (10.5)%. Fibrosis was diffuse in the myocardium surrounding isolated or small groups of myocardial fibres. Frequently, the fibrosis replaced almost all myocardial fibres and resembled microinfarctions.

Confocal Laser Microscopy

Normal hearts (group A)
The myocardial fibres in group A hearts were seen to be attached to each other by several, thin, short, lateral struts and also by long, transverse lateral connections joining groups of myocardial fibres (fig 1D). The interstitial connective fibres were thin and located mainly around vessels.

IDCM hearts (group B)
Group B hearts revealed thin, stretched myocardial fibres, which were enveloped by scarce, thin, and fragmented collagen fibres, and scarce or absent lateral collagen connections, imparting a denuded appearance to the myocytes (fig 1E). There were numerous foci of dense fibrosis, generally free of myocardial fibre.

CCC hearts (group C)
In group C hearts, the myocardium presented large amounts of dense and thick collagen fibres surrounding each myocyte or groups of myocytes that frequently exhibited severe hypertrophy. Dilated vessels were frequently present in the fibrotic zone or adjacent to myocardial fibres (fig 1F).

Study of microvessels
The mean diameters of the arterioles and capillaries, as well as other clinical and pathological data, are shown in table 2.

Normal hearts (group A)
The confocal laser microscopy study and light microscopic analysis revealed that the normal hearts had rectilinear arterioles and capillaries running parallel to the myocytes (fig 2A,E).

IDCM hearts (group B)
IDCM hearts exhibited straightened arterioles and normal capillary vascularisation (fig 2C,G). There were no significant differences in arteriole and capillary diameters between normal and IDCM hearts. Two IDCM hearts had normal mean values and one heart had higher values; however, considerable variation was present in the individual vessel measurements, as reflected by the high standard deviations.

Structural alterations in the arterioles (dilatation of the microvessels and thinning and fibrosis of the media) were seen in histological sections of the IDCM hearts (fig 2D,H), but to a lesser extent than alterations seen in CCC hearts.

CCC hearts (group C)
Group C hearts demonstrated an increased number of perfused capillaries and dilated and tortuous arterioles (fig 2B,F), mainly in the segments distant from the left ventricle apex where the presence of diffuse fibrosis prevented adequate analysis of the microcirculation. The mean arteriole and capillary diameters observed in group C were greater than those observed in normal hearts (p < 0.017) and IDCM hearts (p < 0.008).

The structural alterations (dilatation and fibrosis of media) in the arterioles were greater in the normal human myocardium was 22.4 (3.2). No significant variation was seen among the cases; however, considerable variation was observed from one area to another within the same case (fig 1A). Fibrosis (collagen type I) was scarce, mainly surrounding the vessels. The mean percentage area of fibrosis, assessed by Masson trichrome stain, was 1.2 (1.1)%.

IDCM hearts (group B)
The mean age of patients in group B was 16.0 (17.9) years and the mean heart weight was 394.0 (126.8) g. All subjects showed marked myocardial dilatation. Hypertrophy was mild to moderate. The mean number of lateral connections (struts) was 9.1 (4.08), which is markedly less than the number in normal hearts (p < 0.027). Weaves, coils, and struts appeared to be stretched, fragmented, and poorly stained by silver impregnation (fig 1B). The mean area of fibrosis was 5.9 (6.6)%, less than the area of fibrosis in the group with CCC. As demonstrated by the Kruskal-Wallis test, a significant increase in fibrosis was demonstrated in CCC hearts versus normal hearts (p < 0.01); however no significant difference (p > 0.05) was found between normal and IDCM hearts. The fibrosis in IDCM hearts was usually seen in several foci, generally not enclosing individual fibres or groups of myocytes. Areas adjacent to the fibrotic fields exhibited attenuated myocardial fibres with hypertrophied nuclei and few struts.

CCC hearts (group C)
The mean age of patients in group C was 42.2 (14.3) years and the mean heart weight was 468.0 (62.61) g. The mean number of lateral struts was 15.7 (7.4) and varied considerably. Two hearts exhibited normal mean values and three had lower values (table 1). The remaining struts were frequently thickened (fig 1C). No significant difference was found when compared with normal hearts. The area of fibrosis was 13.8 (10.5)%. Fibrosis was diffuse in the myocardium surrounding isolated or small groups of myocardial fibres. Frequently, the fibrosis replaced almost all myocardial fibres and resembled microinfarctions.
then those observed in IDCM sections. This indicates that these microvessel lesions were chronic and not merely a finding in end stage disease hearts.

**Discussion**

The presence of abnormalities of the myocardial collagen network in the pathogenesis of different dilated heart diseases remains uncertain. CCC has been frequently cited as an example of dilated cardiomyopathy stemming from a known aetiology. However, Weber and colleagues demonstrated the disappearance of struts in thin sections of dilated cardiopathy myocardium. Rossi, using a similar technique, reported a normal number of struts in dilated cardiomyopathy and CCC hearts. Recognising this discrepancy in the literature, we employed a three dimensional microscopic image to analyse microcirculatory and extracellular matrix abnormalities in both entities and, therefore, attempt to establish whether CCC may also be used as a model of IDCM.

In our study, we observed that CCC hearts exhibited severe diffuse fibrosis, encasing individual or groups of myocardial fibres, which probably impairs diastolic function. This was best observed in images derived by three dimensional confocal microscopy. Additionally, the struts were still present as tethering...
groups of myocardial fibres. In IDCM hearts, we observed severe destruction of the struts, and the fibrosis presented with numerous foci, in varying amounts according to each case, and generally did not encase the myocardial fibres. In CCC hearts, in addition to exhibiting the numerous foci of fibrosis and some areas reminiscent of foci of myocardial infarction, also demonstrated a thin and diffuse fibrosis involving individual or small groups of myocytes. The severe hypertrophy present in Chagas’ disease suggests that the evolution of such ventricular dilatation occurs very slowly. The destruction of myocardial fibres and the fibrosis probably contribute to ventricular dilatation and diminished myocardial compliance.

In IDCM hearts, a notably reduced number of struts was seen compared with the number seen in normal hearts, suggesting that myocyte slippage is an important aspect of the development of ventricular dilatation characterising this disease. In most of our cases, the myocardial hypertrophy was not as prominent as the ventricular dilatation. Additionally, the fragmentation and disappearance of struts observed in our cases support the finding of an increased collagen type III ratio in dilated cardiomyopathy hearts by Marijjanowski and colleagues. The statistical analysis showed higher amounts of fibrosis in CCC hearts compared to normal hearts, but not in hearts from the normal group compared to the IDCM group, or in hearts from the CCC group compared to the IDCM group, probably because of the small number of cases and high standard deviations.

The present confocal microscopic study of IDCM hearts demonstrated rectilinear arterioles, which suggests straightening, also probably because of myocyte slippage and ventricular dilatation. Although the mean diameter of the arterioles did not differ from that in the normal group, the measurements from case to case did vary considerably more than in the normal group. Foci of fibrosis in the myocardium are probably related to the fact that end diastolic volumes are increased and end diastolic pressures are high; this likely provides the basis for impaired transmural perfusion. On the other hand, the morphology of the microvessels (for example, some of them straightening with very small lumens) suggests that some vessels may be totally or partially collapsed, causing foci of microinfarctions.

In CCC hearts, severe, diffuse arteriolar dilatation associated with microvessel tortuosity, as demonstrated by confocal microscopy, may cause inadequate balance in the blood flow distribution, worst tissue perfusion (caused by low blood pressure perfusion) in some areas, and multiple infarctions; on the other hand, the fibrotic areas may cause obstructions in the vessel trajectory, favouring deviation of blood flow (a “steal” phenomenon), and appearance of ischemic lesions; the characteristic Chagasic thinning lesions at the apical and basal posterior left ventricle walls may also be the result of ischaemia in the “watershed” between the two main coronary artery branches—the anterior descending and posterior descending arteries and the right coronary and circumflex arteries—caused by low blood pressure perfusion. Chest pain and true myocardial infarction have been described in patients with Chagas’ disease, without coronary obstructive lesions, favouring our hypothesis.

Such microcircular dilatation may be secondary to altered blood flow caused by diffuse fibrosis and the consequent vessel derangement. However, other mechanisms, such as autonomic denervation, endothelial dysfunction, or the delivery of nitric oxide induced by the parasite Trypanosoma cruzi, may also be involved in this arteriolar dilatation. The persistence of T. cruzi antigens has been demonstrated in the late phase of Chagas’ disease and is related to the persistence of inflammatory infiltrate. Such inflammatory infiltrate probably contributes to the fibrosis and arteriolar dilatation. Therefore, in our opinion, the control of parasitism in CCC patients should be important aims.

Microcirculatory changes, such as microvascular spasm microaneurysm and thrombosis, demonstrated in experimentally induced Chagas’ disease were not detected in the present study.

In conclusion, IDCM and CCC have distinct patterns of altered extracellular matrix and microcirculation, which suggest different pathogenic mechanisms of ventricular remodelling. Therefore, CCC should not be considered as an accurate model for IDCM, since these differences may indeed influence therapeutic decisions.

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