Altered patterns of cardiac intercellular junction distribution in hypertrophic cardiomyopathy

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Abstract

Objective—To examine the distribution pattern of intercellular junctions (the mechanically coupling desmosomes and the electrically coupling gap junctions) in hypertrophic cardiomyopathy (HCM) hearts showing myofibre disarray.

Design—Samples from six necropsied hearts were studied, representing the interventricular septum and the free walls of the left and right ventricles. Immunohistochemical labelling of desmoplakin was used as a marker for desmosomes, and of connexin43 as a marker for gap junctions, in single and double stainings. The slides were examined by confocal laser scanning microscopy.

Results—Marked disorganisation of intercalated discs was observed in areas featuring myofibre disarray. Besides overall derangement, localised abnormalities in desmosome organisation were evident, which included: (1) the formation of abnormally enlarged megadiscs; (2) the presence of intersecting disc structures; and (3) aberrant side to side desmosomal connections. Gap junctional abnormalities included: (1) random distribution of gap junctions over the surface of myocytes, rather than localisation to intercalated discs; (2) abundant side to side gap junction connections between adjacent myocytes; and (3) formation of abnormally shaped gap junctions. Circles of myocytes continuously interconnected by gap junctions were also observed. Regions of the diseased hearts lacking myofibre disarray, and control hearts of normal patients and patients with other cardiac diseases, did not show these alterations.

Conclusions—The disorganisation of the intercellular junctions associated with myofibre disarray in HCM may play an important role in the pathophysiological manifestations of the disease. The remodelling of gap junction distribution may underlie the formation of arrhythmogenic substrate, thereby contributing to the generation and maintenance of cardiac arrhythmias associated with HCM.

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Hypertrophic cardiomyopathy (HCM, reviewed in1) is a primary myocardial disease, characterised by a hypertrophied, non-dilated left ventricle, which is not due to coexisting cardiac or systemic disease capable of producing left ventricular hypertrophy, such as valvar disease or hypertension.2 The phenotypic expression and clinical spectrum of the disease are heterogeneous,3 ranging from relatively symptom-free patients4 to those who die suddenly and unexpectedly, characteristically at young age.5 Current evidence implicates the involvement of myocyte structural protein coding genes, including β myosin heavy chain,6 cardiac troponin-T,7 α troponysin,8 and cardiac myosin binding protein C,9,10,11 but the genetic basis of HCM and its relation to expression of the disease phenotype remains to be fully characterised.

A striking histopathological marker of HCM is the presence of myofibre disarray.12,13 This feature of diseased myocardium is characterised by extensive regions of disorganised muscle bundles within which chaotic patterns of interconnection occur between misshapen and hypertrophied cardiomyocytes. These abnormal zones of interconnection between myocytes often give rise to bizarre cross shaped or triangle shaped structures or whorls of cells around a central core of connective tissue. Myofibre disarray is typically most pronounced in the interventricular septum, but it can also be seen in the free wall of the left and even the right ventricle. This cellular disorganisation is not unique to hypertrophic cardiomyopathy, as it can also be found to some degree in other congenital and acquired cardiac diseases14,15; however, extensive and marked myofibre disarray is highly sensitive and specific for HCM.12,13 The presence of myofibre disarray has long been suspected to contribute to the pathophysiological and clinical features of the disease, but to date, the precise consequences of these deranged zones of myocardium are ill defined.

Ventricular cardiac muscle cells of the working myocardium are connected to one another by specialised regions of sarcosomal interaction termed intercalated discs (reviewed in17,18). Each disc contains numerous intercellular junctions of which there are three types, the fascia adherens, desmosome, and gap junction. The fascia adherens and desmosome, which show tightly correlated distribution patterns,19 are both forms of anchoring junction, responsible respectively for attachment of the contractile filaments and the cytoskeleton to sites of adherence between the

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adjoining plasma membranes. The third type of junction—the gap junction—forms the low resistance pathway that enables rapid and orderly flow of cardiac action potential throughout the myofibres, thereby synchronising the mechanical contractions of the heart.

Evidence that gap junctions play a significant part in cardiac pathophysiological mechanisms has recently emerged. In the ischaemic heart, reports that myocyte resting membrane potentials and other electrophysiological characteristics can be essentially normal in the presence of manifest cardiac arrhythmias focused attention on the importance of passive electrical properties. Alterations in gap junction distribution and number have been shown in ischaemic heart disease, and are now considered as key contributory determinants of abnormal conduction properties in the diseased heart. In hypertrophic cardiomyopathy, myofibre disarray leads to profound, histologically visible alterations in the nature of the interactions between cardiac muscle cells, but no investigation has previously been undertaken to determine the remodelling of intercellular junction organisation and distribution that must presumably accompany this process. In the present study, we therefore applied immunolabelling and confocal laser scanning microscopy to investigate the distribution, organisation, and other characteristics of intercellular junctions in HCM hearts featuring myofibre disarray, with particular focus on the gap junction and any alterations that might underlie formation of a proarrhythmic anatomic substrate.

**Methods**

**Tissue samples**

Six hearts obtained at necropsy (age range from 23 to 78 years; three female, three male) were selected from the files of the Departments of Pathology, A Szent-Györgyi University of Medicine, Szeged, Hungary and I Haynal University of Health Sciences, Budapest, Hungary. In four cases, samples were available from four sites: interventricular septum, anterior and posterior free wall of the left ventricle, and free wall of the right ventricle. In each case, at least two different regions, with or without myofibre disarray, were investigated. The material was fixed and embedded in wax according to routine procedures.

Comparable age matched samples from normal human hearts devoid of any cardiac disease and hearts hypertrophied due to aortic stenosis and systemic hypertension were used as control tissues in the study.

**Immunolocalisation of intercellular junctions**

**Desmosome antibodies**

The antibodies used to immunolocalise desmosomes (DP145 and DP121) immunoreact with desmoplakin, a constituent of the desmosomal plaque. DP145 and DP121 are rabbit polyclonal antibodies raised in different rabbits against a trpE bacterial fusion protein containing half of region B and all of region C of the desmoplakin C-terminus. Immunohistochemical and western blot characterisation of the specificity of DP145 for desmoplakins in mammalian epidermal and cardiac tissues are given in Arnemann et al.27

**Gap junction antibodies**

The polyclonal gap junctional antibody (HJ) used against connexin43, the major connexin protein of the working myocardium, was raised in rabbits to a synthetic peptide matching residues 131 to 142 of the cytoplasmic loop of rat connexin43. The characterisation and specificity of HJ for human connexin43 has been described in detail in previous reports from our laboratories. The mouse monoclonal connexin43 antibody used in double staining reactions is raised against a peptide matching the C-terminal amino acid residues 252–270 of rat connexin43 (Zymed Laboratories Inc, South San Francisco, CA, USA). The manufacturer has confirmed antibody specificity by immunoblotting. Specific immunohistochemical labelling of myocardial gap junctions by this probe has been detailed previously by Gourdie et al.28

**Single Immunohistochemical labelling of specimens**

The tissue preparation and labelling procedures were similar for the detection of all three antigens, and were optimised for each antibody. Antigen retrieval procedures were developed in order to optimise immunolabelling of the formalin fixed pathology samples used in this study. These procedures were modified from our published protocols for paraformaldehyde and methanol-fixed tissues. Briefly, sections (5 μm thick) of wax embedded tissue were dewaxed and rehydrated. For antigen retrieval, slides were microwaved in a commercial 800 watt microwave oven at medium power in 0-5 M thiocarbamide buffer (3 × 5 min for HJ, 2 × 5 min for DP145 or DP121) and digested with 0.1% trypsin (Sigma, St Louis, MI, USA) in 0-1% CaCl, and 20 mM Tris (pH 7-4) at room temperature (15 min for HJ, 10 min for DP145 or DP121). The sections were washed with tap water and then treated with 0-1 M L-lysine in phosphate buffered saline (PBS) containing 0-1% Triton X-100. Incubation with primary antibody diluted in PBS at 1:200 for HJ and DP145 or DP121 was carried out overnight at room temperature.

Figure 1 (a) Myofibre disarray in hypertrophic cardiomyopathy featuring disoriented, abnormally shaped myofibres with interstitial fibrosis. Haematoxylin-eosin stain, 134 × . (b) Circular myofibre composed of interconnecting myocytes, around loose central connective tissue. Haematoxylin-eosin stain, 67 × .

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temperature. Sections were then washed with PBS, and biotinylated donkey anti-rabbit secondary antibody (1:250 dilution, Amersham, Buckinghamshire, UK) was applied for 1 h. Slides were rinsed in PBS and treated with streptavidin-fluorescein-isothiocyanate (1:250 dilution, Amersham, Buckinghamshire, UK) for 1 h in the dark at room temperature. After a final wash in PBS, the slides were mounted in Citifluor mounting medium (Citifluor, City University, London, UK).

DOUBLE IMMUNOLABELLING OF GAP JUNCTIONS AND DESMOSOMES
After dewaxing, rehydration, microwaveing, and digestion (see above), the sections were incubated with a primary antibody cocktail, consisting of mouse monoclonal anti connexin43 (Zymed; dilution 1:200) and polyclonal rabbit anti-desmoplakin antibody (Amersham, Buckinghamshire, UK, dilution 1:250) were applied for 1 h. Following another wash in PBS, the sections were incubated in streptavidin-fluorescein-isothiocyanate (DAKO A/S, Glostrup, Denmark, dilution 1:250) for 1 h at room temperature and mounted in Citifluor.

MICROSCOPY OF IMMUNOLABELLED TISSUE
The labelled tissue sections were examined by conventional epifluorescent techniques and confocal microscopy using a Leica TCS 4D confocal laser scanning microscope according to the manufacturer’s instructions (Leica Lasertechnik GmbH, Heidelberg, Germany). In addition to single plane evaluation, optical section series were taken at steps of 1 μm through the depth of the tissue sections. These optical sections were viewed individually or superimposed to reconstruct entire intercalated discs. To correlate the immuno-confocal results with standard histology, identical slides were stained with haematoxylin and eosin using standard procedures for light microscopic evaluation.

Results
STANDARD HISTOLOGY
The tissue samples from diseased hearts disclosed histopathological features typical of HCM. These features included zones of disordered myofibres within which cross shaped or triangle shaped cellular structures were evident (fig 1a). In some regions, the fibres formed circular structures around loose central knots of connective tissue (fig 1b). Areas of interstitial fibrosis, myocardial scarring, and blood vessels with thickened intima were also present.

IMMUNOLABELLING OF INTERCALATED DISCS IN CONTROL “NON-DISEASED” SAMPLES
Immunolocalisation of desmoplakin (desmo-
Figure 4  Altered patterns of gap junction distribution in areas of myofibre disarray in hypertrophic cardiomyopathy. (a) The gap junction immunostaining shows a random spread on myocyte surfaces, instead of being confined to well defined discs (134×). (b) Apparent dissociation between the desmoplakin stained intercalated disc (orange, solid arrow) and the dispersed punctate gap junctions (green, open arrow) (422×). (c) Abnormal side to side gap junctional connections between neighbouring myocytes (arrows, 134×). (d) Abnormally shaped and sized intercalated disc showing abundant gap junction staining. Confocal laser scanning images of anti-connexin43 immunostaining (a, c, d) and anti-desmoplakin (orange) double immunostaining (b).

Hypertrophic cardiomyopathy and intercellular junctions. Confocal Abnormal side spread on immunostaining (b). Figure 1(d). (d) Abnormally shaped and sized intercalated disc showing abundant gap junction staining. Confocal laser scanning images of anti-connexin43 immunostaining (a, c, d) and anti-desmoplakin (orange) double immunostaining (b).

somess) and connexin43 (gap junctions), gave consistent and reproducible staining patterns on standard epifluorescent microscopy. The resolution of fluorescent signal was markedly improved on confocal laser scanning microscopy. In areas of HCM hearts lacking myofibre disarray, and in negative control hearts (that is, "non-diseased" and "normal" myocardial tissues, respectively), junctional molecules immunolocalised mainly at intercalated discs as aggregated domains of bright punctuate fluorescence. In longitudinally sectioned myofibres, immunolabelled discs appeared as sets of short, transversely oriented lines, running almost parallel to each other, as illustrated with anti-connexin43 labelling in fig 2a. On tissue samples in which myocytes had been cross sectioned, the discs appeared as ovoid structures. As reported in detail in our previous studies,29 30 connexin43 immunolabelled gap junctions tended to show a prominent ring of staining at the edge of discs when viewed in this "face on" orientation (fig 2b). Immunolabelled desmosomes in non-diseased control tissues tended to show uniform distributions across intercalated discs.

DISTRIBUTION OF IMMUNOLabelled DESMOSOMES IN AREAS OF MYOFIBRE DIsARRAY Immunostaining of desmosomes in regions of myofibre disarray showed pronounced derangements in the distributions of these junctions. Most desmoplakin immunolabelled discs maintained their transverse orientation with respect to myofibre long axes. However, owing to the chaotic arrangement of myofibres within zones of disarray, the overall distribution of desmosomes at discs was highly disordered (fig 3a and b). In the bizarre triangular or cross shaped myocyte structures common in such zones, the immunolabelling of desmosomes at discs appeared confluent, such that huge megadiscs were formed (fig 3c). Another frequent aberration in the organisation of desmosomes was the occurrence of multilayer discs. Here, prominent immunolabelled discs were observed to intersect at various angles with respect to one another (fig 3c and d). Long, continuous side to side connections were also evident between some myocytes (fig 3d).

DISTRIBUTION OF IMMUNOLabelled GAP JUNCTIONS IN AREAS OF MYOFIBRE DIsARRAY Immunolocalisation of gap junctions in zones of myofibre disarray also revealed marked divergence from normal adult distributions. Despite the overall disorganisation, the pattern of disruption for immunolabelled gap junctions was quite distinct from that observed for desmosomes. In some areas of myofibre disarray, connexin43 immunolabelled gap junctions were no longer wholly confined to well defined foci (that is, intercalated discs; fig 4a and b). Instead, the immunolabelled junctions showed varying degrees of dispersion from the disc over the surface of myocytes. In some instances, this involved the entire myocyte surface (fig 4a), but in other cases, this dispersion of immunolabelled gap junctions was less pronounced. The apparent dissociation between the spatial distribution of the component molecules of mechanical and electrical intercellular junctions is best shown by multiple labelling of the two junction types in the same tissue section (fig 4b). In fig 4b, punctate connexin43 immunolabelling (green) is observed dispersed between myocytes in a distribution that is independent from the disc-like organisation of immunolabelled desmoplakin (orange). Linear arrays of side to side gap junctional interconnections were also frequently observed in longitudinally sectioned cells (fig 4c), and the abnormally enlarged megadiscs also showed abundant connexin43 immunostaining (fig 4d). Interestingly, the circular whorl-like arrangements of cells commonly found in zones of myofibre disarray were comprised of myocytes interconnected by immunolabelled gap junctions. Such structures could be followed through serial sections, and in fortunate orientations, a complete circle of apparently gap junction coupled myocytes could be traced (data not shown).
Discussion

Using immunohistochemistry, with highly specific antibodies and high resolution confocal laser scanning microscopy, our study shows that the distribution pattern of intercellular junctions is markedly altered in HCM hearts featuring myofibre disarray. Both the desmosomes—responsible for mechanical coupling—and the gap junctions—responsible for electrical coupling—disclosed a striking disorganisation. Besides generalised disorganisation affecting larger areas of the myocyte network, specific localised abnormalities were present at the myocyte to myocyte level. The pattern of disorganisation of the intercalated disc staining was confined to areas with myofibre disarray; regions of the diseased hearts lacking myofibre disorganisation showed normal patterns of immunostaining. This suggests that the regionalised derangements of intercellular junctions represent a real pathological process and are not due to postmortem changes or procedural artefacts.

The observed alterations of the desmosomes are likely to have an effect on the passive elastic properties of the HCM myocardium and contribute to diastolic dysfunction, which is a characteristic feature of hypertrophic cardiomyopathy. The long, side to side desmosomal contacts observed would be expected to increase myocardial stiffness, as they cement the plasma membranes of two adjoining myofibres together, thereby probably presenting an obstacle to economic fibre motion. Furthermore, this forced connection might propagatte subtle asynchronies in relaxation through the myocardium.

The pronounced disturbance in gap junction organisation we observed in areas of myofibre disarray suggests a consequent alteration in the passive electrical properties of these fibres, which is a feature of hypertrophic cardiomyopathy. The most striking finding was the dispersion of gap junction proteins over the cell surface, instead of being confined to well delineated intercalated discs. Double staining for desmoplakin and connexin 43 showed, however, that the original structure of the disc is basically intact, as the distribution of the desmoplakin staining remained unaltered; only the connexin 43 staining was laterally dispersed. It is difficult to determine whether these immunolabelled structures represent functional gap junctions or gap junction subunits, disassembled by lateralisation, or internalisation. Whatever the case, the consequence of such an alteration could be a profound change in uniform anisotropy with potential to give rise to arrhythmogenicity. Similar alterations in gap junction distribution have been described in cardiomyopathic hamsters, and in myocytes at the border of healed myocardial infarcts, areas to which the focus of reentry arrhythmias has been localised. Moreover, the observed long side to side gap junction connections, which are absent in normal circumstances, will also presumably lead to decreased anisotropy, opening wide electrical interconnections between neighbouring myocytes and generating the potential for discrete inhomogeneities in the pathways of action potential propagation. Other pathological substrates, which are also likely to form basis for reentry, are the whorl-like myocyte rings, which showed connection by gap junctions all along their circumferences.

It is pertinent that during postnatal growth of rodent ventricular myocardium, gap junctions change from dispersed organisational patterns (that is, similar to those seen in zones of myofibre disarray) to more polarised distributions at intercalated discs. A similar process has been reported from human infants and has been suggested to underpin differentiation of important features of normal cardiac electrophysiology, including anisotropic conduction. HCM can occasionally represent a congenital heart disease in which left ventricular hypertrophy is present during fetal development or shortly after birth, but the increase in wall thickness more commonly occurs during childhood or even develops de novo in adolescence when growth, development, and maturation are accelerated. At present, it cannot be ascertained whether the discrete zones of derangement in electro-mechanical junction organisation observed in HCM result from failure of the maturation process, as the affected myocytes are located within the ventricle or remodelling of previously normal regions of adult myocardium which give rise to patterns of gap junction contact resembling those found in the neonatal heart.

In conclusion, the observed disturbances in intercellular junction distribution may explain some of the pathophysiological features of hypertrophic cardiomyopathy, primarily emphasising the alterations of passive mechanical and electrical properties of HCM hearts. Most importantly, the derangement of gap junction distribution delineate a morphological substrate for arrhythmia generation and propagation. The most striking finding has been the potential to give rise to life threatening ventricular arrhythmias and sudden cardiac death, as frequently seen in patients with hypertrophic cardiomyopathy.

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