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Original article

Human connexin31.9, unlike its orthologous protein connexin30.2 in the mouse, is not detectable in the human cardiac conduction system

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ABSTRACT

In the human heart connexin(Cx)40, Cx43 and Cx45-containing gap junctional channels electrically couple cardiomyocytes, forming a functional syncytium. In the mouse heart, additionally, Cx30.2-containing gap junctions have been detected in the atrioventricular node where they are implicated, together with Cx45, in impulse delay. However, whether the human ortholog of Cx30.2, Cx31.9, is expressed in the human heart has not previously been investigated. We therefore generated Cx31.9 specific antibodies to test for the expression of Cx31.9 in the human heart. These antibodies recognized the Cx31.9 protein in HeLaCx31.9 transfectants by immunofluorescence and immunoblot analyses. However, we did not find punctate Cx31.9 specific immunofluorescence signals in the working myocardium or in the impulse generation and conduction system of adult or fetal human heart. Complementary immunoblot analyses did not reveal Cx31.9 protein in the adult atrial or ventricular myocardium. We conclude that the Cx31.9 protein, unlike its counterpart in the mouse, is not expressed in detectable quantities and is thus unlikely to contribute to the impulse generation and conduction system or the working myocardium of the human heart.

1. Introduction

In the mammalian heart, gap junction mediated coupling between myocytes is required for cardiac development and impulse propagation through the atrial myocardium, conduction system and ventricular myocardium which permits co-ordinated contraction of the atria and ventricles [1,2]. Gap junction channels span the plasma membranes of neighboring cells, thereby allowing the exchange of ions, second messengers and small metabolites along the diffusion gradient [3]. A single gap junctional channel is composed of two hemichannels (connexons), one contributed by each cell, with each hemichannel consisting of six connexin (Cx) protein subunits [4].

Gap junction channels of the human myocardium are comprised of three connexin isoforms, Cx40 (GJA5), Cx43 (GJA1) and Cx45 (GJC1), differentially expressed in distinct regions of the heart [5–8]. In most mammalian species, including humans, Cx43 is highly expressed in atrial and ventricular working myocardium, whereas Cx40 is confined to the atrial working myocardium and the conduction system [7–12]. Cx45 is predominantly expressed in the impulse generation and conduction system, while atrial and ventricular working myocardium express only lower amounts of this connexin [8,13,14].

Alterations in distribution and expression levels of these connexins have been linked to arrhythmia in human cardiac diseases [15,16]. In particular, heterogeneous reduction of ventricular Cx43 is a common feature of human cardiomyopathies as well as cardiac dysfunction and failure of diverse etiology [17–19]. In addition, upregulation of Cx45 has been reported in the failing human ventricle [20], and dominantly inherited mutations in the coding region of the Cx40 gene (*GJA5*) have been linked to the development of atrial arrhythmias such as atrial fibrillation [16]. A range of disparate findings on altered expression levels and distribution of Cx40 and Cx43 have been reported as factors in atrial fibrillation [19,21,22].

In the mouse heart, we recently described an additional cardiac connexin, Cx30.2 (Gjd3) [23]. This newly-discovered cardiac connexin is mainly confined to the cardiac impulse generation and conduction system, specifically, to the sinoatrial (SA) node and the atrioventricular (AV) node. Cx30.2 deficient mice exhibited faster impulse propagation through the AV-node, demonstrating that this connexin is one molecular substrate involved in the slowing of conduction velocity

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within the AV-node [24]. The human ortholog of mouse Cx30.2 is Cx31.9 (GJD3), and this connexin has been reported to be expressed in a range of human tissues, mainly in vascular smooth muscle cells [25,26]. In transfected HeLa cells both these connexins, mouse Cx30.2 and human Cx31.9, exhibit similar electrophysiological properties, i.e. very low unitary conductance and low sensitivity to transjunctional voltage [23,27]. This suggests that Cx31.9 gap junctions in human might have a similar function as Cx30.2 gap junctions in mice. However, whether human Cx31.9, like its mouse counterpart, is expressed in the AV-node, contributing to conduction slowing, or whether it might be expressed in the SA-node or elsewhere in the developing or mature human heart has not previously been investigated.

To this end, we generated polyclonal antibodies directed to two different antigenic epitopes of the C-terminal region of Cx31.9 and investigated the human impulse generation and conduction system as well as working myocardium for the presence of Cx31.9 protein in both adult and fetal hearts. No punctate Cx31.9 specific immunofluorescence signals were apparent in the sinoatrial (SA) node, AV-node, conduction system, or in the atrial and ventricular working myocardium. This suggests that human Cx31.9, unlike its mouse counterpart, plays no role in AV-nodal impulse delay or conduction elsewhere in the human heart.

2. Methods

The investigation conforms to the principles outlined in the Declaration of Helsinki regarding the use of human tissues for research purposes.

2.1. Functional cloning of Cx31.9 and transfection of HeLa cells

The coding region of Cx31.9 was PCR-amplified from human genomic DNA using the upstream primer, Cx31.9KHindIII (5′-CCC AAG CTT GGG CCA CCA TGG GGG AGT GGG C), and one of two downstream primers, Cx31.9BamHISTOP (5′-GCG GAT CCC TAG ATG GCC AGA TCT CGG CG) or Cx31.9BamHIGO (5′-GCG GAT CCG CGA TGG CCA GAT CTC GGC G). The upstream primer included a 5′-HindIII restriction site followed by the optimized translation initiation motif. Both downstream primers contained a 5′-BamHI restriction site and permitted to arrange the presence (Cx31.9BamHISTOP) or the deletion (Cx31.9BamHIGO) of the native stop codon of Cx31.9. A PCR was performed using 1 U of Taq DNA Polymerase (Promega) in reaction buffer containing 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM of each primer and 4% dimethylsulfoxide. PCR-fragments were gel purified, digested with HindIII and BamHI and cloned into the HindIII/BamHI digested vectors pMJ-green [28]. The resulting plasmids, pMJ-Cx31.9 and pMJ-Cx31.9-EGFP, were stably transfected into coupling-deficient HeLa cells.

2.2. Generation of Cx31.9 specific antibodies

Two peptides derived from the middle of the carboxy-terminal domain (ALPSRRPGPEPCAPPA→anti-Cx31.9[A16A]) and the outermost part of the C-terminus (RGKASPATGRRDLAI→anti-Cx31.9[R15I]) were commercially synthesized, coupled to keyhole limpet hemocyanin, and each was injected into two rabbits (Eurogentec, Seraing, Belgium). Sera were affinity purified and characterized using immunoblot as well as immunofluorescence analysis on HeLa cells stably expressing Cx31.9 and Cx31.9-EGFP.

2.3. Human tissue preparation

Human SA-node preparations ($n=3$) were obtained by Dr. Peter Molenaar from The Prince Charles Hospital, Chermside, Australia (Human Research Ethics committee approval EC2565). The work has been ethically approved by the Queensland Government and Manchester University. Samples were from brain dead patients, whose hearts were not used for organ donation, but were available for research. SA-node preparations were snap frozen in isopentane pre-cooled in liquid nitrogen. Tissue sections (30 μm) were cut from the preparations and stained with Masson's trichrome as previously described [29], in order to identify the SA-node, and adjacent sections were immunolabeled as described below.

Human fetal hearts at 9 weeks' gestation were obtained from surgical termination of pregnancy [30].

2.4. Immunofluorescence analyses of HeLaCx31.9 cells and human heart cryosections

HeLaCx31.9 cells grown on glass cover slips (70%–100% confluence) and human heart cryosections were fixed either in ice-cold methanol or in 4% PFA/PBS^-, permeabilized with 0,1% Triton-X in PBS^-, washed in PBS^- and preincubated for 1 h in blocking reagent (PBS^- containing 5% normal goat serum (NGS) or 1% BSA). All slides were incubated overnight at 4 °C or at room temperature with polyclonal anti-Cx31.9 (1:100–1:300), anti-Cx40 (1:500, Y21Y(R968)) [31], anti-Cx40 (1:100, Alpha Diagnostics), anti-Cx43 (1:100, Chemicon) or anti-Cx45 (1:500, Q14E (GP42)) [13,32] antibodies. Connexin localization was visualized using the following secondary antibodies: FITC-swine anti-rabbit (1:25), FITC-donkey anti-rabbit (1:100, Chemicon), Cy3-goat anti-rabbit (1:1000, Jackson) or Cy3-goat anti-guinea pig (1:1000). Slides were washed in PBS^- and cover slips were mounted with aqueous mounting medium. Fluorescent signals were recorded using Leica or Zeiss confocal microscopes. To detect autofluorescence of the human heart tissue which is mainly due to elastic fibers and lipofuscin granules [31], images were taken upon excitation with light of 488 and 594 nm wave length. Subsequently, corresponding images were merged and in the AV-node figure fluorescent signals apparent in both channels were depicted in grey to facilitate the identification of antibody specific signals.

2.5. Immunoblot analyses of HeLaCx31.9 cells and human heart samples

Right atrium, right ventricle, left atrium and left ventricle samples of two human hearts (heart 1: ischemic heart disease; heart 2: healthy heart) [8] (patient no. 4 and no. 36) as well as harvested HeLaCx31.9 and HeLaCx31.9-EGFP cells were lysed in immunoblot loading buffer containing 20% SDS (sodium dodecylsulfate) [13] or Lämmli sample buffer [33], respectively, and scaled up to a final concentration of 10% SDS in all samples. Immunoblot analyses of different HeLa cell lysates were performed as previously described [23]. For Cx43, Cx40 and Cx31.9 immunoblots of human heart samples, 1.25 μg, 2.5 μg or 5 μg total protein lysate were used for gel-electrophoreses (12% polyacrylamide gels), respectively. Proteins were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P), membranes were blocked for 1 h with TBS/0.1% Tween 20/5% skim milk powder and incubated for 2 h at room temperature with the primary antibodies anti-Cx43 (1:1000, Sigma), anti-Cx40 (1:1000, Santa Cruz) or anti-Cx31.9 (1:150) diluted in blocking solution. After three washing steps in TBS/0.1% Tween 20, membranes were incubated with the alkaline phosphatase conjugated goat anti-rabbit (1:10000) or alkaline phosphatase conjugated donkey-anti goat (1:10,000) secondary antibodies for 1 h at room temperature. After additional washing steps, antibody binding was detected using BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitro blue tetrazolium) Color Development Substrate (Promega) according to instructions of the manufacturer.

3. Results

3.1. Cx31.9 specific antibodies and stable HeLaCx31.9 transfectants

In order to examine the expression pattern of Cx31.9, specific antibodies were raised in rabbits. Two immunogenic peptides were