The Gap-Junctional Protein Connexin40 Is Elevated in Patients Susceptible to Postoperative Atrial Fibrillation

Emmanuel Dupont, PhD; Yu-Shien Ko, MD, PhD; Stephen Rothery, BSc; Steven R. Coppen, PhD; Max Baghai, MRCS; Marcus Haw, MS, FRCS; Nicholas J. Severs, PhD, DSc

Background—Atrial fibrillation (AF), a cardiac arrhythmia arising from atrial re-entrant circuits, is a common complication after cardiac surgery, but the proarrhythmic substrate underlying the development of postoperative AF remains unclear. This study investigated the hypothesis that altered expression of connexins, the component proteins of gap junctions, is a determinant of a predisposition to AF.

Methods and Results—The expression of the 3 atrial connexins—connexin43, 40, and 45—was analyzed at the mRNA and protein levels by Northern and Western blotting techniques and immunoconfocal microscopy in right atrial appendages from patients with ischemic heart disease who were undergoing coronary artery bypass surgery. Twenty percent of the patients subsequently developed AF, which allowed retrospective division of the samples into 2 groups, non-AF and AF. Connexin43 and connexin45 transcript and protein levels did not differ between the groups. However, connexin40 transcript and protein were expressed at significantly higher levels in the AF group. Connexin40 protein was markedly heterogeneous in distribution.

Conclusions—Atrial myocardium susceptible to AF is distinguished from its nonsusceptible counterpart by elevated connexin40 expression. The heterogeneity of connexin distribution could give rise to different resistive properties and conduction velocities in spatially adjacent regions of tissue, which become enhanced and, hence, proarrhythmic the higher the overall level of connexin40.

Key Words: arrhythmia • atrial fibrillation • gap junctions

Atrial fibrillation (AF) is a common complication after cardiothoracic surgery, occurring in up to 40% of patients.1–4 The underlying cellular mechanism of AF involves re-entrant circuits resulting from areas of slow conduction and functional conduction block.5 Although this arrhythmia is associated with postoperative sterile pericarditis6 and is more common with advanced age, male sex, and lack of β-blockers,1 intrinsic myocardial factors may also play a role.2,3 Recent discussion has focused on the hypothesis that predisposition to AF may reside in the passive electrical properties of the atrial myocardium,5 ie, the cell-to-cell conduction properties determined by gap junctions.7,8 Gap junctions are clusters of transmembrane channels that link the plasma membranes of neighboring cells, forming the low-resistance pathways for transmitting the action potentials that stimulate contraction.9,10 Each channel consists of 2 hemichannels (termed connexons), and each connexon is a hexamer of connexin subunits.11 In the human heart, myocyte gap junctions may be constructed from up to 3 different connexin isotypes, connexin43, connexin40, and connexin45, which are expressed in a distinctive tissue and chamber-related pattern. An additional isotype, connexin37, is found in vascular endothelial cells.9,12

Connexin43 and connexin45 are expressed throughout the atria at high and low levels, respectively, while connexin40 is expressed at levels 2 to 3 fold higher in the right atrium than the left, reaching levels similar to connexin43 in the former.12 When expressed individually in artificial systems, these different connexin isotypes confer gap-junctional channels with distinctive biophysical properties (eg, unitary channel conductances and permeabilities).13,14 Current evidence suggests that the spatially defined patterns of expression of the different connexin isotypes underlie the precisely orchestrated patterns of current flow that govern normal heart rhythm, and that one potential proarrhythmic factor in the diseased heart is altered connexin expression.9

From this background, we hypothesized that altered connexin expression might contribute to predisposition of surgical patients to postoperative AF. To address this hypothesis, we analyzed connexin expression by immunoconfocal microscopy and Northern and Western blotting techniques in right atrial appendages from patients with ischemic heart disease who were undergoing coronary artery bypass surgery. As expected, a number of patients subsequently developed AF, which allowed a retrospective division of the analyzed...
samples into 2 groups, non-AF and AF. Our findings demonstrated that one connexin type, connexin40, was expressed at significantly higher levels in the group that developed AF.

**Methods**

**Human Samples**

Patients were selected using 3 criteria to obtain a clinically homogeneous group: they all had ischemic heart disease, were undergoing coronary bypass surgery, and had no previous arrhythmic disorder, as assessed by medical history and preoperative ECG. Forty-five samples of the right atrial appendage from 45 patients matching these criteria were collected consecutively over a period of 3 months at the Harefield Hospital National Health Service Trust. Because all the patients were referred, they were managed preoperatively by the referring hospital. At the time of sample collection, the prophylactic use of β-blockers for postoperative AF was not routine practice in the United Kingdom. The project had approval from the institutional ethics committee.

All samples were snap-frozen in liquid nitrogen within a few minutes of collection. This approach permitted total RNA purification for analysis by Northern blotting, extraction of protein in sodium dodecyl sulfate (SDS) buffer for Western blotting, and frozen sectioning of tissue blocks for immunoconfocal analysis. Some of the biopsy samples were too small to permit analysis by all 3 techniques; therefore, we analyzed as many samples as possible in the AF group (7 of 9 samples) using all 3 techniques; the additional 2 samples were therefore, we analyzed as many samples as possible in the AF group (7 of 9 samples) using all 3 techniques; the additional 2 samples were used for Western blotting only. Ten samples of the 36 from the non-AF group were large enough to allow all 3 techniques to be used (4 of 4 samples) using all 3 techniques; the additional 2 samples were used for immunoconfocal microscopy, and use as internal standards throughout.

**TABLE 1. Patient Details**

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<th>Bypass Time, min</th>
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PMH indicates previous medical history; HT, hypertension; MI, myocardial infarction; HC, hypercholesterolemia; NIDDM, non–insulin-dependent diabetes mellitus; β-block, treated with β-blockers before surgery; LA, left atrium; LVEF, left ventricular ejection fraction; CP, cardioplegia; NA, not available; M, male; and F, female.
obtained from a representative non-AF patient (patient 1 in Table 1) in all experiments.

Antibodies
To produce antibodies against connexins 40 and 45, peptides corresponding to residues 316 to 336 of human connexin40, residues 255 to 270 of rat connexin40, and residues 354 to 367 of human connexin45 were used as immunogens in rabbit (connexin40) and guinea pig (connexin45). Antiseras were affinity-purified against their respective peptide. Characterization of the anti-connexin40 [S15C(R83)] and anti-connexin45 [Q14E(GP42)] antibodies has been reported previously. Characterization of the new human connexin40 antibody [designated Y21Y(R968)] developed for the present study is reported in Results. For connexin43, a commercially available mouse monoclonal antibody (Chemicon) was used due to its established specificity. Controls in immunological experiments (Western blot, immunofluorescence, and immunogold) were (1) omission of the primary antibody and (2) preincubation (1 hour) of the diluted antibody with 100 μg/mL of the relevant peptide.

Protein Extraction and Western Blotting
For Western blotting, frozen, pulverized tissue was lysed in a solution containing 20% SDS (10 μL for each milligram of frozen powder). Four micrograms of total protein per lane were run on 12.5% SDS polyacrylamide gels and electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P). The resulting replica was incubated with anti-connexin antibody and then with appropriate alkaline phosphatase-conjugated secondary antibodies (goat anti-mouse IgG for the anti-connexin43 and donkey anti-rabbit IgG for the anti-connexin40). The enzymatic activity was revealed using nitroblue tetrazolium and BCIP substrate solution. Quantification of Western blots was done with densitometric scanning. Linearity of optical density was verified by loading a range of total protein amounts and scanning the resulting immunolabeled membrane. To relate connexin to the myocytic compartment (given that different samples will contain variable quantities of blood proteins), the same samples (8 μg per lane) were run in a parallel gel, stained with Coomassie blue, and densitometrically scanned. The values obtained for myosin were used to normalize the values obtained with the anti-connexin43 and the anti-connexin40. Data are expressed as a percentage of the signal obtained from the same representative control patient (patient 1; run as a standard internal control in all experiments) as in the Northern analysis.

Immunofluorescence Confocal Microscopy
Frozen sections (10 μm) on glass coverslips were fixed in methanol, incubated with the anti-connexin antibody of choice and with the appropriate secondary antibodies (CY3-conjugated donkey anti-rabbit IgG to detect the anti-connexin40, donkey anti-mouse IgG to detect anti-connexin43, or goat anti-guinea-pig IgG to detect anti-connexin45), and mounted. Immunolabeled sections were examined using a Leica TCS 4D confocal microscope. Images were recorded using triple-channel scanning (CY5, CY3, and fluorescein isothiocyanate fluorescence) and were transformed into projection views of optical sections taken at 0.5 μm intervals.

Immunogold Electron Microscopy
Samples for postembedding, immunogold, thin-section electron microscopy were fixed (2% paraformaldehyde in PBS), dehydrated in an ethanol series, infiltrated and embedded in Lowicryl K4 mol/L, and polymerized with ultraviolet light. Ultrathin sections on nickel grids were incubated at room temperature successively in 1% BSA in PBS, 1% gelatin in PBS, 0.02 mol/L glycine in PBS, connexin40 antibody, PBS, and 10-nm gold-conjugated anti-rabbit antibodies. The sections were then washed with PBS, fixed (1.25% glutaraldehyde), washed with distilled water, dried, and stained with uranyl acetate and lead citrate before examination in a Philips EM301 electron microscope.

Statistical Analysis
All analysis was done using GraphPad Prism 2.01 (GraphPad Software). Experimental and clinical data were compared using an unpaired, 2-tailed Mann-Whitney test. Statistical differences were judged significant at P≤0.05.

Results
Patients
Of the original 45 patients used in the study, 20% developed symptomatic AF. The arrhythmia was detected by 12-lead ECG (either routine or emergency). All the patients in the AF group were in sustained AF (Table 2). Statistical analysis by 2-tailed Mann-Whitney test for age, number of grafts, cross-clamp time, bypass time, preoperative and postoperative serum potassium concentrations, P-wave duration, left atrial size (assessed by M-mode echocardiography), and left ventricular ejection fraction (assessed by either M-mode echocardiography or ventriculography) showed no significant differences between the non-AF and AF groups (P>0.05). The small number of patients studied may explain the lack of significant differences for some of the clinical factors.

Connexin40 Antibodies
A new antibody against the human isoform of connexin40 [designated Y21Y(R968)] was developed specifically for immunofluorescence and Western analysis of connexin40 in this study. The antibody was characterized by Western blotting (Figure 1A) and immunoelectron microscopy (Figures 1B and 1C). Western blot analysis demonstrated positive labeling of a distinct 40-kDa band in tissues known to express

<table>
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<th>TABLE 2. Arrhythmia Duration and Ventricular Response in AF Group</th>
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high levels of this connexin; low or no connexin40 signal was seen in tissues expressing negligible connexin40. A 65-kDa band was present in all heart samples but absent from lung samples. There was no relationship between the intensity of the 40-kDa band and that of the 65-kDa band. Peptide inhibition completely abolished the labeling of both bands. Y21Y(R968) gave identical labeling patterns to our alternative anti-connexin40 antibody [S15C(R83)], with no signal found in the working ventricular myocardium distant from the endocardial surface and positive labeling of endothelial cells and Purkinje fibers. Immunogold labeling showed that no structures other than gap junctions were labeled. These data indicate that the 65-kDa band results from cross-reactivity with another protein unrelated to gap junctions that was not present in the lung.

Immunoconfocal Analysis of Connexins 40, 45, and 43

The human atrium is rich in elastic fibers and in lipofuscin, which are strongly autofluorescent over a wide wavelength range; this impairs the visualization of immunofluorescent signals when single-channel recording is used for confocal microscopy. Therefore, we used 3-channel recording and combined the image data to generate color images in which lipofuscin appears white (strong emission in all wavelengths), elastin as green (stronger emission in the fluorescein channel), and connexin labeling (CY3 fluorochrome) as red (stronger emission in the rhodamine channel). Using the Y21Y(R968) or the S15C(R83) antibodies, connexin40 was consistently localized as prominent punctate labeling between atrial myocytes and in the endothelium of intramural arteries (Figures 2A and 2B). With both antibodies, the distribution of connexin40 labeling was heterogeneous in all samples, with regions of myocardium (up to a few millimeters in size) displaying little staining adjacent to other areas that were intensely labeled (compare Figure 2A with Figure 2B or left side with right in Figure 2B). Endothelial connexin40 labeling did not reveal any detectable differences between or within samples. The labeling patterns for the other myocyte connexins (45 and 43) were as previously reported.12 Connexin43 labeled at high fluorescence intensities similar to those observed for connexin40 (Figure 2C), whereas the level of connexin45 labeling was much lower than that of the other connexins (Figure 2D). Both connexins 43 and 45 were homogeneously labeled at the myocyte intercalated disks. Connexin37 was present exclusively in endothelial cells. Scoring of slides by visual inspection for the level of labeling for each connexin type did not reveal detectable differences between the 2 groups.

Northern Blotting Analysis

Figure 3 shows a typical Northern analysis for connexins 40, 43, 45, and 37. All the probes for the different connexins labeled a single mRNA band at the size expected from previous reports.12,15 Exposure times for the gels in Figure 3A were 140 hours for connexin45 (first hybridization), 15 hours for both connexin40 and connexin43 (second and third hybridizations, respectively), and 170 hours for connexin37 (fourth hybridization). From the exposure times using probes of similar sizes and specific activities, connexin40 and connexin43 transcripts appeared to be present in similar amounts. Quantification and comparison of the data from patients who developed AF with those who did not are shown in Figure 3B. Band intensities were normalized with the values for the 18S and were expressed as a percentage of the value obtained for the internal standard (patient 1) run in all gels. Connexin40 mRNA was, on average, ≈50% higher in the atria of patients who subsequently developed postoperative AF than in those who did not (P<0.002). On an individual basis, by setting a threshold in the overlapping range between the 2 groups, a high connexin40 transcript content identified >75% of the patients prone to AF (top of Figure 3B). The amounts of the other connexins were not significantly different between the groups (P>0.05).
Figure 2. Low magnification of atrial samples immunofluorescently labeled for connexin40 (Cx40; A and B), connexin43 (Cx43; C), and connexin45 (Cx45; D). Connexin staining is seen as red fluorescence, autofluorescence of elastic fibers as green, and autofluorescence of lipofuscin granules as white-purple or white-red (L indicates lipofuscin). Connexin40 is present at intercalated disks, which is seen in various orientations from transverse to en face (inset in A), and in endothelial cells of intramural arteries (* in A and B). Distribution is somewhat heterogeneous, as seen in B, where left side of field displays prominent labeling and right side shows sparse labeling. Connexin43 signal is prominent (C) but, in contrast to connexin40, is absent from arteries (indicated by * in C; identified by ring of green fluorescence of medial elastic fibers). Connexin45 is present only in low amounts (D); the much higher power of laser setting necessary to record connexin45 signal thus results in much brighter intensity of lipofuscin fluorescence in D compared with A, B, and C.
Western Blot Analysis

Connexin40 and connexin43 were detected as single bands at \( \sim 40 \) and \( \sim 43 \) kDa, respectively (Figure 4). Similar loading of myocytic protein was not feasible because all the samples still contained an unknown but large amount of blood and the actin/myosin ratio was not constant. Therefore, to normalize the Western blot values, we used the values for myosin (because actin is a major component of the cytoskeleton in other cell types). As shown in Figure 4B, the connexin40 protein signal was significantly higher in the AF group than in the non-AF group (\( P=0.028 \)), whereas connexin43 was expressed at similar levels in both groups. As with the Northern analysis, setting a threshold in the overlapping point between the groups identified \( >75\% \) of the patients prone to AF. The correspondence of the results obtained by Northern and Western analysis was examined by plotting the individual mRNA values against the corresponding protein values, followed by linear regression analysis. These results (connexin40: \( r^2=0.5291, \ P=0.0009 \); connexin43: \( r^2=0.26 \),

Figure 3. A. Representative Northern blot analysis of total RNA extracted from patients in non-AF (1 to 5; CONT) and AF groups (6 to 14; AT FIB). The same membranes have been hybridized with cDNA for connexin (Cx) indicated. Position of wells, 28S rRNA, and 18S rRNA are shown. Each probe labels a single band at expected molecular weight. Bottom panel is a hybridization with an oligonucleotide specific for 18S rRNA used to assess equivalent loading and to normalize values obtained with specific probes. B, Scatter graphs showing data for connexin mRNA levels measured in 2 groups. All values are expressed as a percentage of value obtained for patient 1 to allow standardized comparison of different experiments. Only connexin40 levels were significantly different (\( P=0.002 \)) between non-AF (C, *) and AF groups (†). Horizontal line in connexin40 graph in B indicates threshold for \( >75\% \) negative and positive prediction.

Figure 4. A. Typical Western blot analysis of heart protein homogenate for connexin40 (Cx40, top), connexin43 (Cx43; middle), and Coomassie blue (Coo; bottom) staining of samples (Coomassie blue was used to assess gel loading and to normalize data with values obtained by densitometric scanning for myosin). Non-AF patients (1 to 5; CONT) are shown on left immunoreplica; patients 1 and 2 were repeated on right immunoreplica for comparison with AF group (6 to 14; AT FIB). Position of separating gel (arrow) and molecular weight standards (MWS) are indicated. Connexin40 migrates at \( \sim 40 \) kDa. Anti-connexin43 antibody labels a single band at \( \sim 43 \) kDa, thus representing highly phosphorylated form of this protein; some lower molecular weight bands just below are also weakly labeled and represent low amounts of dephosphorylated or unphosphorylated forms. Scatter graphs of connexin40 and connexin43 protein levels are shown in B. Signals were normalized with values from Coomassie blue-stained myosin and are expressed as a percentage of value for patient 1. Only comparison of connexin40 levels between non-AF and AF groups was significant (\( P=0.029 \)). Numbers on left in A indicate molecular weight in kDa.
earlier report also noted a lack of connexin40 in neonatal heart, some distinctive species differences have also emerged. For example, unlike the human, goat, and mouse atrium, connexin40 is undetectable in the rat atrium. Although an

Discussion

There is a well-recognized link between the development of AF and sterile pericarditis after open heart surgery, but the underlying electrophysiological substrate that favors the development of AF in some patients but not others is unclear. The present study involves a retrospective analysis of a relatively small number of patients; however, our data show that 1 of the 3 connexins in atrial myocytes, connexin40, is expressed at significantly higher levels in patients who develop postoperative AF than those who do not. The higher levels of connexin40 transcript found in AF patients are mirrored by higher levels of connexin40 protein (demonstrated by quantitative Western blotting) and, hence, the enhanced expression could potentially lead to functional differences in conduction properties. A conspicuous feature of connexin40 protein distribution in the human atrium is its heterogeneity, potentially resulting in spatially adjacent regions of atrial myocardium having markedly different resistive properties and, hence, conduction velocities. These features could combine to enhance susceptibility to the development of re-entrant circuits as, the higher the overall level of connexin40, the more extreme would be such differences between the spatially adjacent regions.

Our present findings in patients contrast with those reported in animal models. Lack of connexin40 was reported to result in increased atrial vulnerability to arrhythmia in the mouse. That heterogeneity of atrial connexin40 protein distribution might form a cellular substrate favoring AF was previously suggested from a goat model of sustained AF. However, in contrast to the goat model, in which the connexin40 heterogeneity was reported to result from the rapid pacing used to induce AF, the heterogeneity observed in human atria was consistently present in both the non-AF and AF groups. Whether such heterogeneous connexin40 distribution is a normal feature of the human atrium or a consequence of a natural or pathological process (eg, aging or ischemia) is unclear. In the human situation, an investigation of connexin expression at the time of arrhythmia, as was done in the goat, is precluded.

Although many species share common features in the tissue-specific pattern of connexin isoform expression in the heart, some distinctive species differences have also emerged. For example, unlike the human, goat, and mouse atrium, connexin40 is undetectable in the rat atrium. Although an earlier report also noted a lack of connexin40 in neonatal human atrium, unpublished work from our laboratory (R. Kaba et al, unpublished data, 2000) reveals that this connexin is abundantly expressed in the human heart during embryonic development. Against this background, current knowledge does not permit assessment as to whether the present findings have relevance to young patients undergoing cardiac surgery. The postnatal period (up to the age of 6 years in the human) is certainly marked by progressive changes in the organization of connexin43 gap junctions in the ventricular myocardium, culminating in the formation of discrete intercalated disks, and atrial connexin expression patterns may well be similarly dynamic during this period. An assessment of connexins in relation to arrhythmia in the younger patient requires a more detailed analysis of connexin expression patterns during human development.

Although no difference was apparent in the levels of connexin43 and connexin45 between the 2 groups, that these 2 connexins are simultaneously expressed is also relevant to the potential functional effects of differing connexin40 levels. Studies from in vitro expression systems indicate that gap junction channels made from different connexin types have distinctive conductance properties, and mounting evidence suggests that coexpression of connexins in intact tissues gives rise to a range of modified channel properties compared with those of homomeric, heterotypic channels. Such multiple connexin expression is compatible with a wide range of different molecular arrangements of connexins, including heterotypic channels, heteromeric connexons, and mixtures of different types of homotypic channels. The functional correlates of the multitude of possible molecular arrangements cannot be predicted at present, but in vitro systems manipulated to mirror the multiple in vivo connexin expression patterns, which are amenable to functional and structural analysis, are currently being developed to clarify this issue.

The linear regression results suggest that connexin40 levels in the heart are regulated largely by the transcript steady-state level, as was previously reported for connexin43. The finding that different connexin40 levels occur while connexin43 levels remain constant suggests that where the 2 connexins are coexpressed (ie, atria and conductive tissues), they are regulated independently. If distinct “factors” or regulatory pathways that alter the expression of each connexin independently could be identified, then the possibility of developing specific therapeutic interventions based on the modulation of selected connexins exists. It could also be possible to exploit measurements of connexin40 to identify patients susceptible to postoperative AF before symptoms develop.

Acknowledgments

Supported by the Wellcome Trust (grant 04628/Z/950) and the European Commission (grant QLRT-1999–00516).

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_Circulation_. 2001;103:842-849
doi: 10.1161/01.CIR.103.6.842

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/103/6/842

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