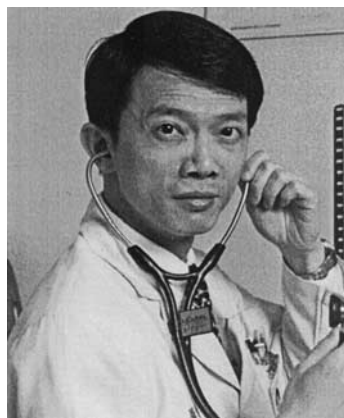


# Alteration of gap junctions and connexins in the right atrial appendage during cardiopulmonary bypass

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**Objectives:** We investigated the influence of cardiopulmonary bypass on cardiomyocyte gap junctions and connexins.

**Methods:** Samples were collected at intervals during operation from the right atrial appendage in 21 patients (mean  $\pm$  SD) age  $55 \pm 21$  years). Immunodetection of connexins was conducted by Western blotting and confocal microscopy with parallel electron microscopic examination of gap junctions.

**Results:** Downregulation of connexin 43 during the course of operation occurred in more than half of the patients. The mean densitometric value of connexin 43 decreased by 23%, with samples from patients with coronary artery disease showing a greater reduction than seen in those from patients with other diseases ( $31\% \pm 22\%$  vs  $10\% \pm 24\%$ ,  $P = .04$ ). Such alterations were confirmed by confocal microscopy, which also demonstrated reduced connexin 45 immunolabeling in most patients. Electron microscopy revealed a reduction in the dimensions of cell membrane-located gap junctions and more frequent intracytoplasmic gap junctional membrane in samples from later time points ( $P = .04$ ).

**Conclusions:** Downregulation of connexins accompanied by a reduction in gap junctions is common in the cardiomyocytes of the right atrial appendage during cardiopulmonary bypass. The association of a marked reduction in connexin 43 with coronary artery disease may imply inadequate intraoperative cardiac protection in patients with this disease.

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**P**ropagation of action potentials between cardiomyocytes is mediated via gap junctions, constructed from connexin proteins.<sup>1</sup> In human beings, the working atrial cardiomyocyte mainly expresses connexin (Cx) 40, Cx43, and Cx45.<sup>2</sup> Physiologically, gap junctions are dynamic structures; the half-lives of the component connexins are reported to be between 1 and 3 hours.<sup>3,4</sup> Thus, the expression and distribution of connexins may undergo alterations within short periods. Among factors affecting cardiac gap junctions, ischemia is known to have a profound effect.<sup>5,6</sup>

In vitro studies have shown that several factors related to ischemia, such as elevated proton and decreased adenosine triphosphate levels, suppress gap junctional communication function.<sup>7-9</sup> Investigation of coronary artery disease (CAD) has also demonstrated that gap junction distribution and connexin expression in the ischemic zone are disturbed.<sup>5,6,10</sup> This raises the possibility that cardiomyocyte gap junctions may change during cardiopulmonary bypass (CPB), when the heart faces a stress similar to the challenge of ischemia.

Although efforts have been introduced to ameliorate the ischemic insult during cardiac surgery (eg, application of hypothermia and cardioplegic solution<sup>11</sup>), the heart is not perfectly preserved, as is evident from the fact that more than 90% of patients display postoperative ventricular dysfunction.<sup>12</sup> Another major factor affecting the clinical outcome, the occurrence of postoperative arrhythmia,<sup>13</sup> is also

TABLE 1. Clinical profiles and analysis of Cx43 and Cx40

Patient No.	Age (y)	Sex	Diagnosis	CP	Cx43 (%)*	Cx40 (%)*
1	25	F	ASD	C	86	74
2	43	F	ASD, MVC	B	32	41
3	16	M	ASD, MVC, VSD	B	97	73
4	34	F	ASD, PAPVR	C	104	20
5	72	M	Endocarditis	B	96	130
6	43	M	Mitral regurgitation	C	93	103
7	75	M	Valvular aortic stenosis	B	109	44
8	10	M	VSD, PS	C	102	72
9	44	M	CAD (DVD)	B	68	95
10	43	M	CAD (TVD)	B	48	109
11	63	F	CAD (TVD)	B	102	141
12	66	M	CAD (TVD)	B	74	58
13	67	F	CAD (TVD)	B	61	91
14	70	F	CAD (TVD)	B	49	69
15	71	M	CAD (TVD)	B	53	97
16	71	M	CAD (TVD)	B	30	63
17	72	F	CAD (TVD)	B	82	57
18	78	F	CAD (TVD)	B	78	50
19	78	M	CAD (TVD)	B	83	60
20	46	M	CAD (TVD + LM)	B	110	118
21	60	M	CAD (TVD + LM)	B	64	137

ASD, Atrial septal defect; MVC, mitral valve cleft; VSD, ventricular septal defect; PAPVR, partial anomalous pulmonary venous return; PS, pulmonary stenosis; DVD, double vessel disease; TVD, triple vessel disease; LM, left main artery disease; CP, type of cardioplegic solution; C, crystalloid, 1 L contains Na 120 mmol/L, Cl 180 mmol/L, K 36 mmol/L, Ca 2.4 mmol/L, Mg 32 mmol/L, bicarbonate, 10 mmol/L, albumin 1.25%, mannitol 0.4%, methylprednisolone 500 mg; B, 1:1 mixture of blood and crystalloid.

\*Percentage of the value of the total connexin amount of the latest sample against that of the initial one; see "Methods" section for definition of the direction of change of Cx43 and Cx40 between the initial and last sample.

related to the bypass procedure.<sup>11</sup> Because coordination of contractile function between cardiomyocytes relies on gap junctions and disturbance of the connexins is reportedly linked to arrhythmogenesis,<sup>14</sup> it is of interest to examine the influence of CPB on gap junctions and connexins. To this end, we set out to investigate the temporal expression pattern of cardiomyocyte gap junctions and connexins during the course of cardiac surgery.

## Methods

### Samples

Ethical approval was granted by the institutional review board of the Mackay Memorial Hospital, Taipei, Taiwan. Informed consent was obtained from each patient. The procedures followed were in accordance with institutional guidelines. Human heart samples were collected in the operating theater from patients undergoing cardiac surgery and were taken from the right atrial appendage bordering the inserted cannula linked to the extracorporeal oxygenator. The first sample was collected just before or within 5 minutes after initial infusion of cardioplegic solution. Subsequent samples, one for each time point, were taken at intervals of 20 to 40 minutes. For each patient, 2 to 3 samples were collected. Each sample, measuring about 8 mm<sup>3</sup>, was bisected or trisected and allocated for immunoblotting, immunolabeling, and in some cases also thin-section electron microscopy.

### Immunodetection of Cardiomyocyte Gap Junctions

**Anti-connexin antibodies.** Two affinity-purified, well-characterized connexin isotype-specific polyclonal antibodies were used to detect Cx40 (raised in rabbits, Y21Y[R968]) and Cx45 (raised in guinea pigs, Q14E[GP42]).<sup>1,15,16</sup> For Cx43, a mouse monoclonal antibody was purchased (CHEMICON International, Inc, Temecula, Calif).

**Immunoblotting.** Western blotting was conducted as described previously elsewhere.<sup>2</sup> A 20- $\mu$ g aliquot of sample was loaded in each lane. The membranes were incubated for 60 minutes with the anti-Cx43 (1:500) or anti-Cx40 (1:100) antibody, washed, and incubated with an alkaline phosphatase-conjugated secondary antibody (1:5000; Pierce Chemical Company, Rockford, Ill). The color reaction was carried out with nitro blue tetrazolium/5-bromo-1-chloro-3-indolyl phosphate (NTB/BCIP) solution (Pierce).

**Immunolabeling.** Cryosections 10  $\mu$ m thick were immunostained for each of the connexins, as described previously elsewhere.<sup>2</sup> Immunostained samples were examined by confocal laser scanning microscopy with a Leica TCS SP (Leica, Heidelberg, Germany).

### Analysis

Densitometric scanning and analysis were performed on immunoblots. For Cx43, bands of various phosphorylated forms in each sample were measured separately and the values were added together to represent the total amount. When the values of samples

from the same patient differed by more than 10%, the samples were considered different. The dimensions of gap junctions found in ultrastructural examination were also measured. For this purpose, 25 randomly selected fields from each sample, each of which possessed at least 1 gap junction, were recorded at 25,000 $\times$  magnification. For each patient, 2 samples of different time points were examined. Mean ( $\pm$  SD) values of the length of gap junctions per field were obtained for each sample. Data were compared statistically by the *t* test.

## Results

Samples were collected from 21 patients (age  $55 \pm 21$  years, male/female ratio 13:8). The clinical profiles of these patients are summarized in Table 1.

### Analysis of Western Blots

Alterations in Cx43 and Cx40 during the course of observation were found to occur in most patients (Figure 1), although the patterns of change differed between these two connexins. For Cx43, the total amount remained unaltered in 8 cases, whereas it decreased in the other 13 participants. As a whole, the mean value of total Cx43 signal of all participants decreased by 23% (Table 1). A conspicuous feature of the Cx43 Western blots was a variable degree of phosphorylation. For Cx40, in 12 patients later samples had lower Cx40 levels than did the initial samples, whereas in 5 patients the Cx40 levels at consecutive time points were similar. On the other hand, in 4 patients the later samples had higher Cx40 levels (Figure 1, *B*). These variable patterns may reflect the marked heterogeneity of Cx40 distribution that was apparent in the atrial samples.

Considering the relationship between the changes in the connexins and the clinical profiles, it was found that total Cx43 amount declined more in samples from patients with CAD than in those from other diseases ( $31\% \pm 22\%$  vs  $10\% \pm 24\%$ ,  $P = .04$ ).

### Immunoconfocal Microscopy

Cx43 was found abundantly and homogeneously distributed in samples taken at the initial time point (Figure 2, *A*). In the later samples, the expression of Cx43 remained similar in patients with consistent total Cx43 amount during the bypass, whereas the signal was unevenly decreased in patients showing a decline in total Cx43 amount (Figure 2, *B*). By contrast, Cx40 varied in abundance and distribution in all samples (Figure 2, *C* and *D*), which made the comparison difficult between samples of different time points from each patient. For Cx45, although the signal was faint, the distribution and change resembled those of Cx43 in each patient (Figure 2, *E* and *F*).

### Electron Microscopic Examination

Ultrastructural examination, conducted in samples from 7 patients (cases 9, 10, 11, 14, 17, 18, and 20), showed that

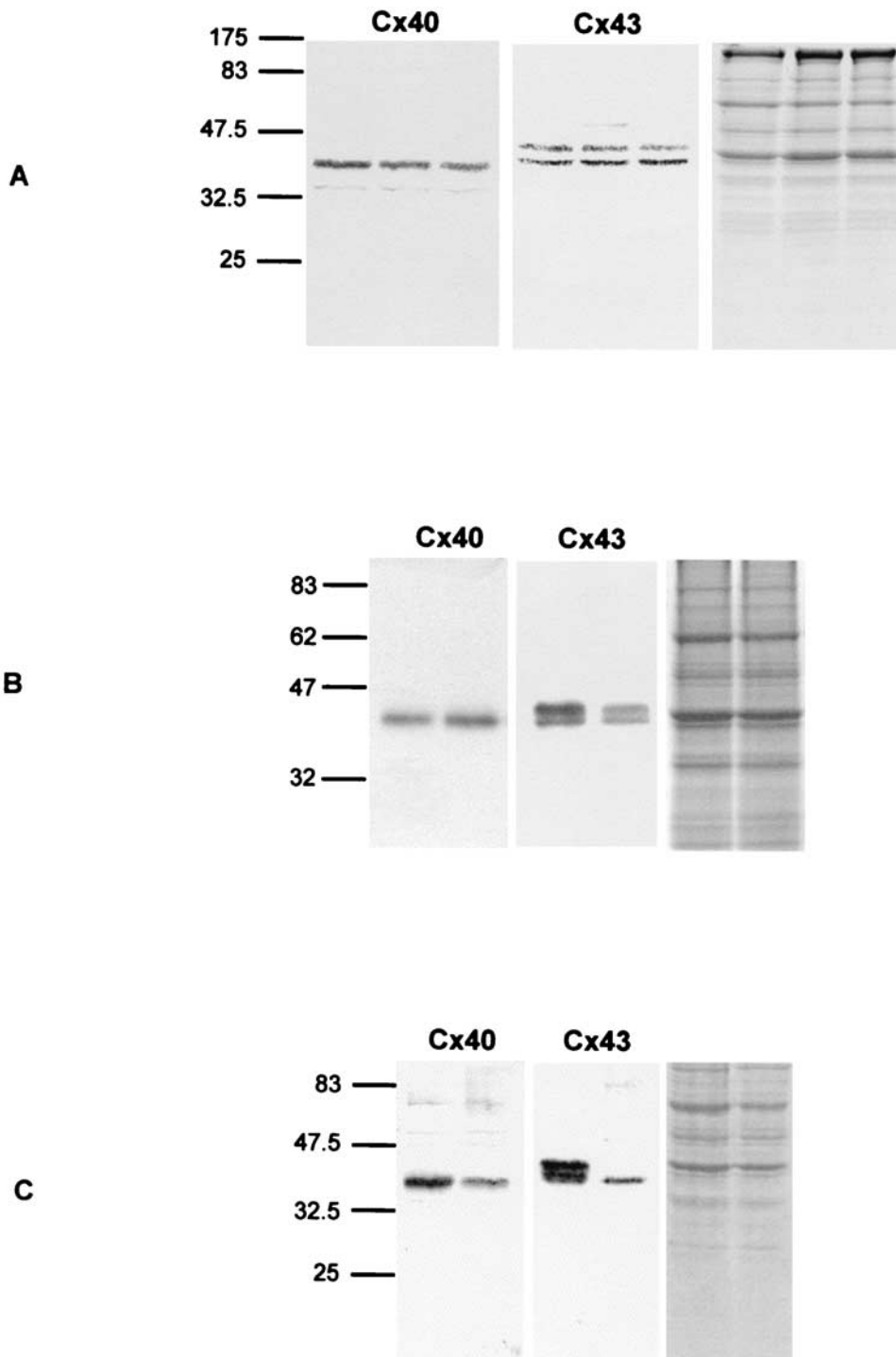
gap junctions varied in location and morphologic characteristics, appearing as the typical linear structures incorporated into the cell membrane (Figure 3, *A*), as intracytoplasmic annular forms (Figure 3, *A* and *B*), or as linear structures stacked in parallel within the cell (Figure 3, *C* and *D*). The intracytoplasmic forms of later time points tended to have a less well-defined pentalaminar structure (Figure 3, *B-D*). Comparison of the cell membrane-located gap junctions between samples of different time points revealed that the initial samples had more gap junctions per field ( $1.6 \pm 0.6$  vs  $1.2 \pm 0.5$ ,  $P = .11$ ) of significantly larger size (total gap junction length  $600 \pm 338$  nm vs  $341 \pm 172$  nm,  $P = .03$ ) than did the later time points. In contrast, the intracytoplasmic gap junctions were more numerous at the later time points ( $0.1 \pm 0.1$  vs  $0.3 \pm 0.2$ ,  $P = .04$ ).

## Discussion

In this study, downregulation of gap junctions in the right atrial appendage was a common phenomenon during CPB. In addition, patients with CAD showed a particularly marked decline in Cx43. These findings suggest that the normal life cycle of cardiac gap junctions is disturbed during cardiac anesthesia.

During cardiac surgery under certain circumstances different portions of the heart are not equally well protected. For example, in the presence of CAD, the stenotic lesions impair the delivery of cardioplegic solution to its territory. This may explain the more marked reduction of Cx43 in our patients with CAD. It should also be borne in mind that during such operations, the right atrial appendage (the source of samples of this study), which is located in the upper part of the heart and directly exposed under the illuminating beam, may not be constantly immersed in the cooling solution. The possibility for local rewarming and less satisfactory preservation than occurs with other parts of the heart may exist, although whether as a result the atrial appendage is more vulnerable to connexin and gap junction alterations than other parts of the heart remains unclear.

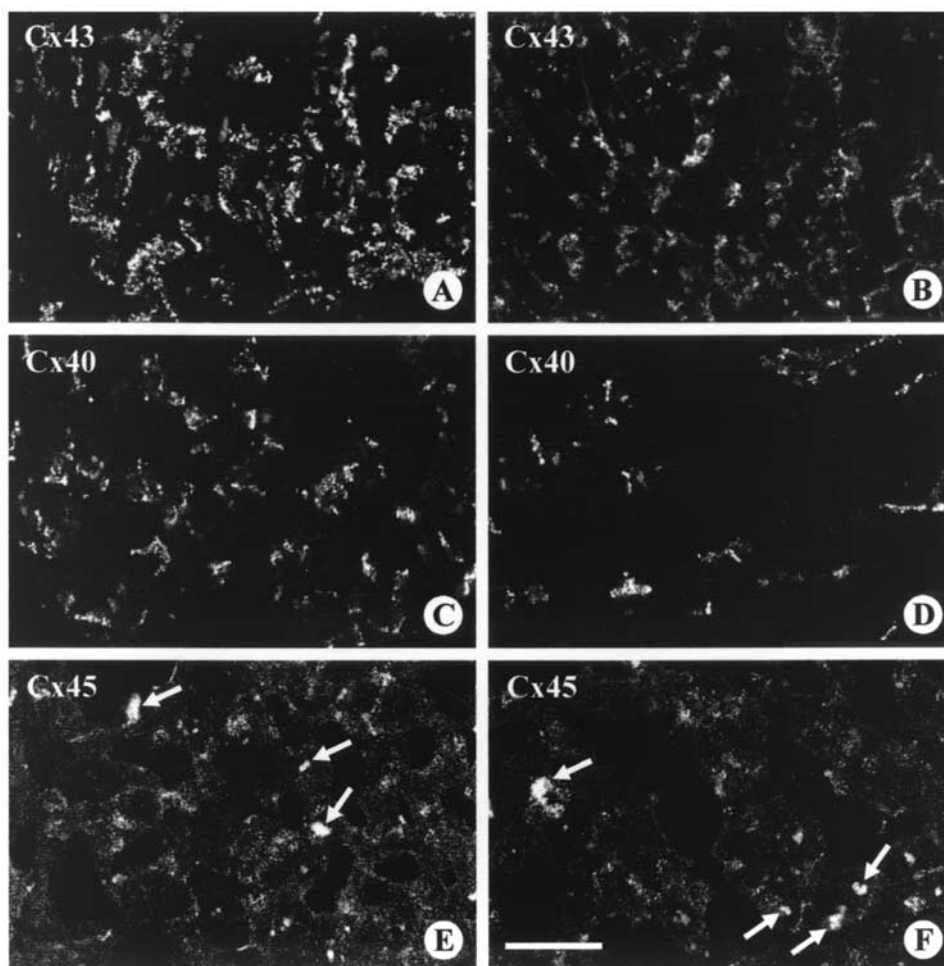
The downregulation of Cx43 within a short time observed here by Western blotting and immunoconfocal microscopy is striking. The electron microscopic results explain how this process may be mediated. One mechanism for degradation of gap junctions involves first withdrawal of gap junctional membrane from the cell membrane into the cytoplasm, followed by proteolysis.<sup>4</sup> Previous studies with thin-section electron microscopy have shown that internalized gap junctions (those removed from the cell membrane) have distinct morphologic characteristics, such as annular shapes and parallel membrane stacks.<sup>4,17</sup> Our electron microscopic results showed that gap junctions possessing these characteristics were more frequently observed in samples from later time points, than in those from the initial ones, suggesting that withdrawal of gap junctions from the cell membrane is enhanced. This may also explain the highly



CSP

**Figure 1.** Analysis of connexin proteins by Western blotting. Lanes in each membrane, of which connexin detected is denoted at top, are arranged chronologically so that *left* is loaded with initial sample and *right* with last sample. **A**, In case 3, Cx40 follows stepwise decline from *left* to *right*. By contrast, both phosphorylated and nonphosphorylated forms of Cx43 in each lane possess similar density. **B**, In case 21, Cx40 is higher and Cx43 is lower at later time point. **C**, In case 2, lower levels of Cx40 and Cx43 are seen at later time point. Gel stained with Coomassie blue (right membranes) shows equal loading in each lane.





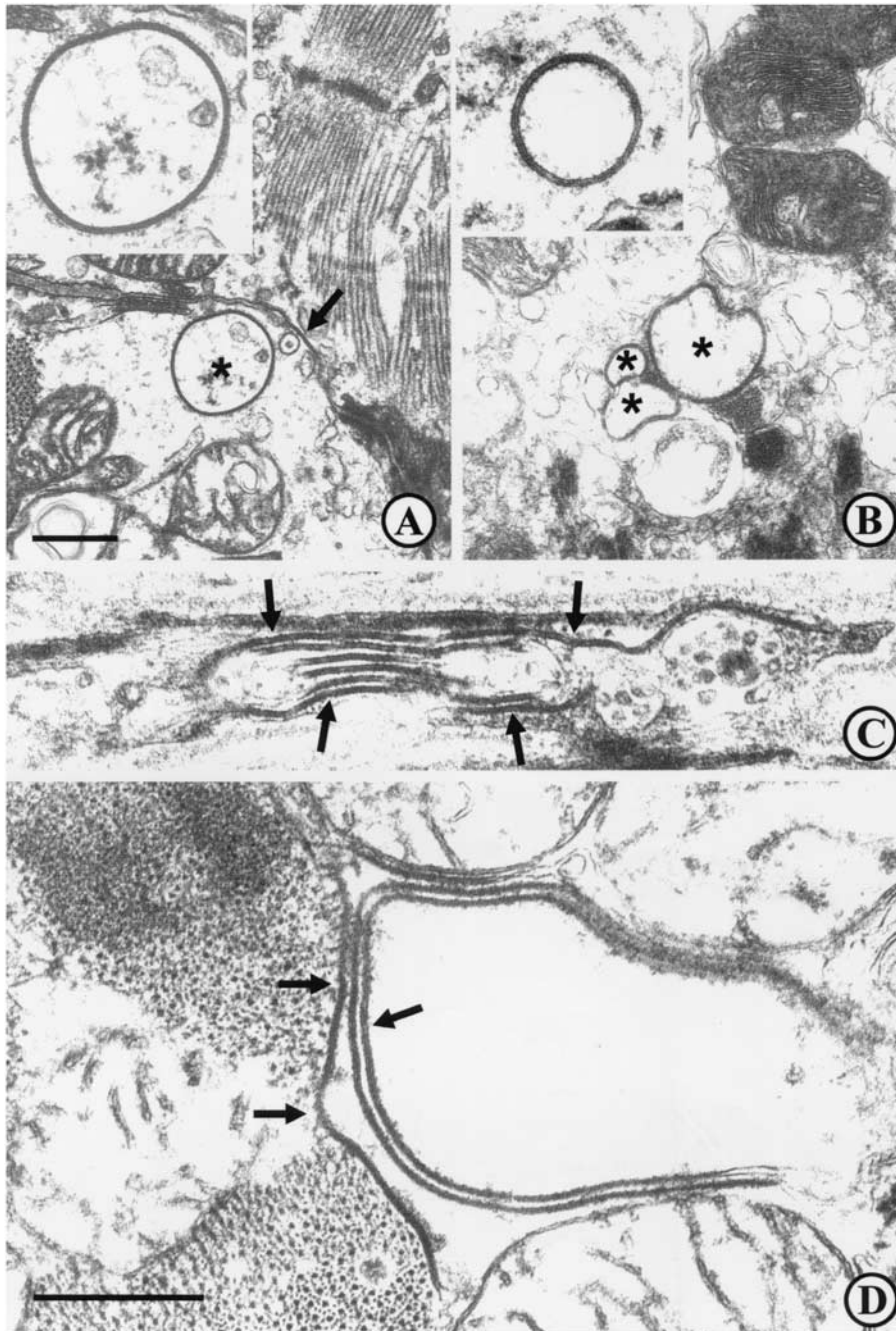
**Figure 2.** Immunofluorescence images demonstrating typical changes of expression of each connexin during CPB. Images on *left* are taken from samples of initial time point, whereas those on *right* are from latest time point. **A** and **B**, Images from case 10 demonstrate downregulation of Cx43 along course. **C-F**, Images from case 16 also demonstrate downregulation of Cx40 and Cx45 along course. Note autofluorescent spots from lipofuscin in images **E** and **F** because of enhanced laser power. *Bar* represents 30  $\mu\text{m}$ . (Original magnification  $\times 400$ .)

variable degree of dephosphorylation of Cx43 observed in most samples, because removed gap junctions are likely to be targeted for degradation into the lysosomal compartment.

For Cx45, the small quantity of this connexin in the tissue precluded its detection with the Q14E(GP42) antibody by Western blotting; nevertheless, the immunofluorescence results show that reduction of Cx45 signal was common, as was seen for Cx43. On the other hand, unlike Cx43 or Cx45, Cx40 did not show an apparent reduction during the operation. It is important to bear in mind that Cx40 in the human atrial tissues is markedly heterogeneous in distribution, as observed in this and previous studies.<sup>16</sup> Thus, data from small samples, such as those used in this study, may overestimate or underestimate the average content of Cx40, and the finding that the level of Cx40 expression apparently

fluctuated during the bypass should be interpreted cautiously.

Downregulation of gap junctions and connexins during heart surgery may contribute to the cardiac depression observed in the initial postoperative period as a result of the lack of proper contractile coordination between individual myocytes as well as such other recognized contributors as inflammation triggered by reperfusion.<sup>18</sup> This possibility depends on the quantity of gap junctional channels lost, the extent of cardiac muscle involved, and the recovery rates of connexin synthesis and gap junction formation. In addition, reports from connexin gene knockout studies and animal models of myocardial infarction indicate that reduced or heterogeneous expression of connexins provides a substrate for the development of arrhythmia.<sup>14,19</sup> Furthermore, the



**Figure 3.** Thin-section electron micrographs demonstrating intracytoplasmic gap junctions in cardiac samples taken early (A) and late (B-D) during CPB. A, Case 10; annular gap junction (*asterisk*) is present adjacent to cell border where membrane bound gap junction (*arrow*) is situated. *Inset* shows enlarged view of annular gap junction with its characteristic pentalaminar structure. B, Case 17; cluster of three annular gap junctions (*stars*). *Inset* shows enlarged view of annular gap junction taken from another sample (case 9). C, Case 18; nine linear gap junctions of variable length stacked in parallel (between *arrows*). Note that pentalaminar morphology is less clearly defined but still can be recognized along portions of the junctions. D, Case 7; three linear gap junctions gather closely (between *arrows*). Images A and B are at the same magnification; Images C, D, and all the insets are at the same magnification. *Bars* represent 0.4  $\mu\text{m}$ . (Original magnifications: A and B,  $\times 6000$ ; insets, C, and D,  $\times 25,000$ .)

formation of scar tissues around the atriotomy site after operation affects action potential propagation. A multitude of factors thus may act in concert to lead to the onset of postoperative arrhythmia.

In conclusion, cardiomyocyte gap junctions and their component connexins in the right atrial appendage appear to be altered during CPB. Further investigation is required to clarify the clinical significance of our findings.

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