Individual Gap Junction Plaques Contain Multiple Connexins in Arterial Endothelium

Hung-I Yeh, Stephen Rothery, Emmanuel Dupont, Steven R. Coppen, Nicholas J. Severs

Abstract—Gap-junctional intercellular communication in endothelial cells is implicated in the coordination of growth, migration, and vasomotor responses. Up to 3 connexin types, connexin40 (Cx40), Cx37, and Cx43 may be expressed in vascular endothelium according to vascular site, species, and physiological conditions. To establish how these connexins are organized at the level of the individual endothelial gap junction, we used affinity-purified connexin-specific antibodies raised in 3 different species to permit double and triple immunolabeling in combination with confocal and electron microscopy. Using HeLa cells transfected with Cx37 and Cx40 for characterizstion, the anti-Cx37 antibody (raised in rabbit) and the anti-Cx40 antibody (raised in guinea pig) were shown to recognize single bands of 37 and 40 kDa, respectively, on Western blots and to give prominent punctate labeling at the cell borders, specifically in the corresponding transfectant. By applying these antibodies together with mouse monoclonal anti-Cx43 for double and triple immunofluorescence labeling at confocal microscopy, rat aortic and pulmonary arterial endothelia were found to express all 3 connexin types, whereas coronary artery endothelium expressed Cx40 and Cx37 but lacked Cx43. High-resolution en face confocal viewing of the aortic endothelium after double labeling demonstrated frequent colocalization of connexins, with distinct variation in the expression pattern within a given cell, where it made contact with different neighbors. Triple immunogold labeling at the electron-microscopic level revealed that aortic endothelial gap junctions commonly contain all 3 connexin types. This represents the first definitive demonstration of any cell type in vivo expressing 3 different connexins organized within the same gap-junctional plaque. (Circ Res. 1998;83:1248-1263.)

Key Words: gap junction n connexin n endothelium n immunoconfocal microscopy n freeze-fracture cytochemistry

Coordination of cellular responses at the endothelial interface between the blood and underlying tissues is mediated by multiple signaling mechanisms, including direct intercellular communication via gap junctions. Among the functions in which endothelial gap-junctional intercellular communication has been implicated are the migratory behavior of endothelial cells after injury, angiogenesis, endothelial growth and senescence, and the coordination of vasomotor responses.1–5 Gap junctions comprise clusters of transmembrane aqueous channels linking the cytoplasmic compartments of neighboring cells.6–8 These channels, insulated from extracellular space, provide electrotonic and metabolic pathways for the direct cell-to-cell transfer of small-signaling molecules and ions. Each channel is made from 2 connexons, 1 contributed from each of the partner cells, with each connexon comprising a hexamer of 6 connexin molecules. The connexins belong to a multigene family of related proteins, at least 13 members of which have been identified in mammals.6,9 Many cell types and tissues are now documented to express more than 1 connexin type, and functional studies using in vitro expression systems indicate that gap junction channel properties differ according to the component connexin present.7,9–13 Multiple expression of connexins in a given tissue or cell type could in theory result in different populations of gap junctions, each containing a single connexin type, or gap junctions in which mixtures of connexins are present in the form of heterotypic channels, different types of homotypic channels, or heteromeric channels.6,9 In view of the disparate channel properties associated with the different connexin types, there is considerable current interest in determining precisely how cells that express multiple connexins actually model their gap junctions in vivo.

Our knowledge of the connexin composition and organization of endothelial gap junctions has so far remained limited, however. Studies by Northern blotting, Western blotting, and immunocytochemistry indicate that connexin37 (Cx37), Cx40, and Cx43 are expressed in the vascular wall, and various studies have detected 1 or more of these connexins at the mRNA or protein level in specific types of endothelial cell.2,14–23 However, the available data in any given endothelial cell type are fragmentary, with the various reports differing in the precise connexin profiles identified.
Furthermore, although a series of studies has examined connexin expression in cultured endothelial cells, these studies have been largely confined to Cx43, and culturing per se generally leads to profound alterations in the usual in situ pattern of connexin expression.\textsuperscript{5,24–26} Although the evidence suggests that some endothelia may express Cx40, Cx43, and Cx37 in situ,\textsuperscript{20,21,23} no studies have yet established whether an individual endothelial cell of a given vessel type can produce all 3 connexin types, and if so, whether Cx40, Cx37, and Cx43 are assembled into separate or the same gap junctions.

We recently established that the pattern of expression of Cx37, Cx40, and Cx43 shows distinctive variations in different segments and subzones of rat arteries, and that in aortic endothelium Cx40 and Cx43 may colocalize to the same gap-junctional plaque.\textsuperscript{23} To investigate the pattern of connexin expression at the level of the individual gap junction in endothelium that express 3 connexin types, we have designed and characterized 2 new antibodies to Cx37 and Cx40, specifically for application in multiple-labeling experiments. These antibodies were applied to survey connexin expression by immunoconfocal microscopy in selected endothelium in rat arteries and, using the aorta, were applied in a new high-resolution approach to confocal viewing of endothelium en face in combination with double labeling for pairs of the 3 connexin types. Using the same custom-designed antibodies for triple immunogold labeling at the electron-microscopic level, we show by means of thin-section and freeze-fracture cytochemistry that gap junctions of the rat aortic endothelium typically contain all 3 connexin types. This represents the first definitive demonstration of any cell type in vivo expressing 3 different connexins organized within the same gap-junctional plaque.

Materials and Methods

Tissue Preparation

Specimens of aorta, coronary artery, and main pulmonary artery were obtained from adult male Sprague-Dawley rats (weight, 330 to 445 g). For cryosectioning, 5 rats were killed by dislocation of the neck, and the heart, aorta, and main pulmonary artery were quickly removed. After rinsing with PBS containing 10 \( \text{mM} \) heparin, the heart was bisected and the arterial tissues were cut into 5-mm-thick transverse arterial rings for rapid freezing in isopentane at –160°C. In a separate procedure, used for immunoelectron microscopy and for whole mounts examined en face by confocal microscopy, 5 rats were perfused with formaldehyde (freshly prepared from paraformaldehyde). These animals were anesthetized by intraperitoneal injection of midalozam-fentanyl citrate/fluaniou sideline and perfused retrogradely, via a catheter in the abdominal aorta, with heparinized PBS followed by PBS containing 2% paraformaldehyde (pH 7.4) for 15 to 20 minutes. For the en face confocal work, 5-mm-thick transverse rings of the fixed thoracic aorta were frozen in isopentane and stored under liquid nitrogen before en bloc immunolabeling. For immunoelectron microscopy, small specimens (1 mm\(^2\)) were processed for low-temperature embedding in the acrylic resin Lowicryl K4 M (Polysciences Ltd). All animal procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986.

Generation and Affinity Purification of Anti-Cx37 and Anti-Cx40 Antibodies

Peptides corresponding to residues 266 to 281 of rat Cx37 (YLPMGEFPSSPPCPTY) and to residues 256 to 270 of rat Cx40 (VQGLTPPPDFNQCLK) were synthesized as immunogens for the production of antibodies in rabbits and guinea pigs, respectively. The Cx37 peptide was a gift from Dr J-P. Briand (Institut de Biologie Moleculaire et Cellulaire de Strasbourg, Strasbourg, France), and the antibodies against this sequence were raised by Elevage Scientifique Des Dombres. For the Cx40 sequence, peptide synthesis and antibody production were done by Research Genetics Inc. From the series of antisera produced in different animals, that shown for each peptide to produce optimal results in trials was selected for use. These antisera, designated Y16Y(R4) (anti-Cx37) and V15K(GP319) (anti-Cx40), were affinity purified against the appropriate peptide coupled to an activated chromatography matrix (epoxy-activated Sepharose 6B (Pharmacia Biotech) for Cx37 antisera, and UltraLink-immobilized dianinodipropylamine (Pierce) for Cx40 antisera). The antibodies were eluted from the column using 0.1 \( \text{mM} \) glycine, pH 2.4 (for anti-Cx37), or 0.1 \( \text{mM} \) glycine/2% acetic acid, pH 2.9 (for anti-Cx40), and immediately neutralized with 1 \( \text{mL} \) Tris. The eluted antibodies were then desalted by using a 10DG desalting column (Bio-Rad) equilibrated with PBS. Glycerol was added to 50% and azide to 0.02%, and the resulting antibody solutions were stored at –20°C.

Characterization of Anti-Cx37 and Anti-Cx40 Antibodies by Western Blotting

The specificity of the purified anti-peptide antibodies was tested by Western blotting and immunolabeling using HeLa cell transfectants expressing murine Cx37 (HeLa-37), Cx40 (HeLa-40), and wild-type cells (HeLa-W).\textsuperscript{10} These cell lines were kindly donated by Prof Klaus Willecke (University of Bonn, Bonn, Germany). The nontransfected cells were cultured in DMEM (GIBCO-BRL) supplemented with 10% FBS (GIBCO-BRL), 100 \( \text{U/mL} \) penicillin, and 100 mg/mL streptomycin (GIBCO-BRL). The transfected cells were cultured in the same medium but with the addition of 0.5 \( \mu \text{g/mL} \) puromycin (Sigma Chemical Co). Cell cultures were maintained at 37°C in a moist atmosphere of 5% \( \text{CO}_2 \).

Alkaline membrane preparations of cultured cells were prepared by scraping the cells into protein buffer (0.5 \( \text{mL} \)/100 cm\(^2\)), 10 \( \text{mM} \) Tris, pH 7.5, 10 \( \text{mM} \) iodoacetamide, 10 \( \text{mM} \) tetrasodium pyrophosphate, 10 \( \text{mM} \) EDTA, 20 \( \text{mM} \) sodium orthovanadate, 2 \( \text{mM} \) phenylmethylsulfonyl fluoride, 1 \( \text{mM} \) leupeptin, and 1 \( \text{mg/mL} \) peptatin A. The cell suspension was then made alkaline by the addition of 0.7 \( \text{mL} \) 40 \( \text{mM} \) NaOH and put on ice. The suspension was sonicated for 20 seconds at power 3 (microprobe, W-380 sonicator from Heat Systems-Ultrasonics, Inc.) and centrifuged at 23 000 \( \times g \) for 30 minutes at 4°C. The supernatant was removed and the pellet washed with protein buffer and resuspended in a solution of 20% SDS/10 \( \text{mM} \) EDTA in 0.1 \( \text{mM} \) Tris, pH 6.8\textsuperscript{27}: with brief sonication.

Ten micrograms of each sample per lane, as assessed using the Bio-Rad DC protein assay, was run on 10% SDS-polyacrylamide gels and transferred to Immobilon-P transfer membrane (Millipore) at constant voltage (30 V) overnight using the Bio-Rad minitransblot system. The membrane was blocked with 0.1% Tween in Tris-buffered saline (20 \( \text{mM} \)) Tris and 150 \( \text{mM} \) NaCl, pH 7.5) containing 5% fat-free milk, incubated for 1 hour at room temperature with the affinity-purified anti-Cx37 (1:30) or anti-Cx40 antibody (1:50) in Tris-buffered saline/0.1% Tween/1% BSA, washed, and incubated with the appropriate alkaline phosphatase-conjugated secondary antibody (donkey anti-rabbit Ig, 1:5000, Pierce; rabbit anti–guinea pig, 1:1000, Zymed). After washing, detection of alkaline phosphatase activity was carried out using a freshly diluted solution of nitroblue tetrazolium/5-bromo-1-chloro-3-indolyl phosphate (Promega) in 0.1 \( \text{mM} \) Tris, 0.1 \( \text{mM} \) NaCl, and 5 \( \text{mM} \) MgCl\(_2\), pH 9.5. Phosphate inhibition controls were done by adding peptide to the primary antibody solution (100 \( \mu \text{g/mL} \)) 30 minutes before incubation.

Immunofluorescence Labeling of Gap-Junctional Connexins

In addition to the affinity-purified anti-Cx37 and anti-Cx40 antibodies, Cx43 was localized using a commercially available mouse
Multiple Connexins in Endothelial Gap Junctions

monoclonal antibody (directed against residues 252 to 270 of rat Cx43; Chemicon). The secondary antibody/detection systems used to visualize immunolabeled connexons were donkey anti-rabbit, anti-guinea pig, and anti-mouse IgG conjugated to either CY3 or CY5 (Chemicon). For single labeling of individual connexons, CY3-conjugated antibodies were used. For double labeling of 2 connexins, 1 CY3-conjugated antibody and 1 CY5-conjugated antibody were used in combination. Where triple labeling (3 connexin types) was technically feasible, CY3-, CY5-, and FITC-conjugated secondary antibodies were used. In selected experiments in which 1 or 2 connexins were visualized, simultaneous endothelial cell marking was carried out using rabbit anti-human von Willebrand factor polyclonal antiserum (anti-VWF) detected with donkey anti-rabbit-FITC (Dako).

Single and multiple labeling was carried out on 3 types of specimens: transfected HeLa cells, tissue sections, and intact aortic rings. HeLa cells grown on glass coverslips and 10-μm-thick cryosections mounted on poly-L-lysine-coated slides were immersed in methanol at −20°C (5 minutes), rinsed in PBS (5 minutes), and blocked in 0.5% BSA in PBS (15 minutes). The perfusion-fixed aortic rings were rinsed in PBS (5 minutes) and treated in 0.1% Triton X-100 in PBS (15 minutes) before blocking in 0.5% BSA in PBS (15 minutes). Incubation with the anti-connexin antibody of choice followed the same procedure for all types of specimens. The optimal conditions determined and subsequently routinely used for each antibody were as follows: Cx37 antibody (dilution 1:60) at room temperature overnight, Cx40 antibody (dilution 1:100) at room temperature for 1 hour, and Cx43 monoclonal antibody (1:1000) at room temperature overnight. The samples were then treated with CY3-conjugated secondary antibody (1:250, room temperature, 1 hour). In experiments in which 2 of the 3 connexins were simultaneously localized in the same samples, sequential incubation with each of the anti-connexin antibodies was followed by incubation with a mixture of the 2 secondary antibodies (CY3, and CY5: 1:250). Where simultaneous marking of endothelial cells was carried out in sections, incubation with anti-VWF (1:500, room temperature, 1 hour) followed by swine anti-rabbit IgG-FITC (1:25, 1 hour) was then carried out. Between each step of immunolabeling, the samples were given a thorough wash in PBS. For processing the aortic rings, all steps were carried out in 2-mL Eppendorf tubes so as to protect the delicate endothelial surface from mechanical damage while maintaining ease of accessibility for retrieving the specimens.

For triple labeling to allow simultaneous detection of the 3 connexins (feasible only on coronary artery sections), the optimal procedure was to treat sections first with a mixture of the Cx37 (1:100) and anti-Cx40 (1:1000) antibodies (room temperature, overnight), followed by anti-Cx43 antibody (1:1000, 37°C, 2 hours). The sections were then treated with a mixture of anti–rabbit IgG-CY3 (1:250), anti–guinea pig IgG-CY5 (1:250), and anti–mouse IgG-FITC (1:25) at room temperature for 1 hour.

After the secondary antibody/detection procedure, the cells on coverslips, and the tissue sections, were mounted directly using Citifluor mounting medium (Agar Scientific Ltd). For en face viewing of whole mounts, the aortic rings were carefully cut open and placed on slides with the luminal side up before being mounted similarly.

All experiments included positive and negative controls using HeLa cell transfectants. Additional negative controls also conducted on animal tissue sections and rings were (1) omission of secondary antibody and (2) inclusion of the peptide (20 to 100 μg/mL). Each secondary reagent was confirmed to be species specific by secondary antibody crossover experiments (eg, mouse primary antibody followed by anti-rabbit and/or anti–guinea pig secondary antibody; rabbit primary antibody followed by anti-mouse and/or anti–guinea pig secondary antibody; etc). Each primary antibody was thus tested against all the secondary antibodies used during multiple labeling, and cross-reactivity was excluded.

Confocal Laser Scanning Microscopy and Analysis of Colocalization

Immunolabeled sections were examined by confocal laser scanning microscopy using a Leica TCS 4D equipped with an argon/krypton laser and fitted with the appropriate filter blocks for the detection of FITC, CY3, and CY5 fluorescence. The images were taken using single-, dual-, or triple-channel scanning and transformed into projection views using sets of 5 consecutive single optical sections taken at 1-μm intervals. All specimens were examined within 24 hours of immunolabeling. For presentation of the images, combinations of “false” colors that optimized visualization of the multiply labeled structures of interest in the digital images were selected. As red and green stand out most clearly, they are the colors of choice for illustrating gap junction labeling, particularly where superimposition (colocalization) of signals occurs (this gives an easily visualized yellow signal). Blue was most suitable for generalized marking where a less conspicuous signal suffices (as in endothelial marking) and was also used as the third color when 3 connexins were simultaneously localized in the coronary artery.

The extent of connexin colocalization in the endothelium of the different arteries was analyzed in sections that had been double labeled for (1) Cx37 and Cx40, (2) Cx40 and Cx43, and (3) Cx37 and Cx43. Images were collected by simultaneous dual-channel scanning using the ×63 objective lens with zoom factor 1.0, and projections were split into the 2 separate (“split”) images corresponding to each of the connexin types. Fifty immunolabeled spots were randomly selected from each double-label image, and the component connexins of each spot were determined by analyzing the split images. When a spot visible on the double-label image had a corresponding spot on each of the 2 split images (ie, both connexins were present), it was classified as showing colocalization. If a spot appeared on just one of the split images, and not on the other, it was classified as containing one or other of the individual connexin types. Because Cx37 showed a patchy distribution, portions of endothelia expressing no Cx37 were not included as we sought to determine whether, when Cx37 was present, other connexin(s) were present in the same junctional spot. We excluded Cx43 labeling of smooth muscle cells by analyzing immunolabeled spots on the luminal side of the internal elastic lamina only. For each artery type, 5 images from each animal (ie, a total of 1250 gap junction spots per artery) were analyzed. Results were expressed as mean percentages (±SD) of gap junction spots showing fluorescence for each individual type of connexin that also showed fluorescence for the second connexin type, in each double-label combination. The data were compared statistically by t test.
Postembedding Immunogold Labeling for Electron Microscopy

For immunogold labeling, samples were embedded at low temperature in Lowicryl K4 M. Paraformaldehyde perfusion-fixed aortic samples were dehydrated in 30% ethanol at 4°C for 30 minutes, followed by 50% and 70% ethanol at −20°C for 30 minutes and 1 hour, respectively, and then 90% and absolute ethanol (3 times) at −30°C (1 hour for each step). Infiltration was at −30°C for periods of 1 hour with 1:1 and 2:1 mixtures of Lowicryl K4 M:ethanol, followed by pure Lowicryl (Agar Scientific Ltd) overnight. After a further incubation in pure resin for 1 hour, the samples were embedded in fresh pure resin in gelatin capsules and polymerized with UV light at −30°C for 16 hours and then at room temperature for up to a further 72 hours, using a Balzers FSU 010 low-temperature embedding unit. Ultrathin sections were processed for single and multiple labeling. For single labeling, the sections were incubated at room temperature overnight with anti-Cx37 (1:30), anti-Cx40 (1:100), or anti-Cx43 (1:500) antibodies in PBS containing 0.5% BSA, followed by incubation with the appropriate 10-nm-diameter gold/goat anti-rabbit, anti–guinea pig, or anti-mouse secondary antibodies (BioCell) diluted 1:50 in PBS for 1 hour. For triple labeling, sections were first treated with anti-Cx37 (1:30) at room temperature overnight followed by incubation with a mixture of anti-Cx40 (1:100) and anti-Cx43 (1:500) at room temperature for 4 hours. These sections were then incubated with a mixture of 5-nm gold/anti-rabbit, 10-nm gold/anti–guinea pig, and 15-nm gold/anti-mouse complexes (1:50 for each) for 1 hour. Double labeling was carried out using sequential primary antibody treatment under similar conditions. Controls consisted of omission of primary reagents and using each primary antibody with all 3 secondary antibodies. Each secondary reagent was confirmed in this way to be specific to the appropriate primary antibodies. The labeled sections were examined with a Philips EM301 electron microscope.

Freeze-Fracture Cytochemistry

Immunolabeling of freeze-fractured aortic endothelial gap junctions was carried out using a method modified from that of Fujimoto. In this freeze-fracture cytochemical technique, conventional freeze-
fracture replicas are first prepared; the biological material is then digested using SDS. The SDS removes the bulk of the biological material, leaving a fine layer of proteins adherent to the replica, which may then be localized by immunogold labeling.

Small samples of unfixed rat aorta were cut into 3-mm-thick rings, bisected, and placed briefly in 25% glycerol in PBS. Samples were mounted, endothelial cell side up, on gold flat-topped carriers, and picked up on copper 460-mesh grids for electron-microscopic examination.

**Results**

**Characterization of Anti-Cx37 and Anti-Cx40 Antibodies**

Western blot analysis demonstrated that the anti-Cx37 antibody recognized a single band at 37 kDa in HeLa-37 cell membrane preparations (Figure 1A). This band was absent in corresponding preparations of HeLa-W and HeLa-40 cells. Similarly, the anti-Cx40 antibody recognized a prominent 40-kDa band only in HeLa-40 cell membranes and not in HeLa-W or HeLa-37 cells (Figure 1B). Labeling of these bands was abolished by preincubation of each antibody with the peptide to which it was raised.

Immunoconfocal microscopy of HeLa-37 cells incubated with the anti-Cx37 antibody and of HeLa-40 cells incubated with the anti-Cx40 antibody revealed conspicuous, irregularly distributed punctate labeling at cell-cell borders (Figure 2A and 2B). In both cases, labeling was abolished by peptide inhibition. No positive signal was apparent when the Cx37 antibody was tested on HeLa-40, HeLa-43, or HeLa-W cells (Figure 2C, 2E, and 2G) or when the Cx40 antibody was tested on HeLa-43, HeLa-W cells (Figure 2D, 2F, and 2H).

**Localization of Multiple Connexins by Confocal Microscopy**

Double labeling of sections of the 3 arterial types revealed some similarities but also distinct differences in the relative abundance of signal and extent of colocalization of the 3 connexin types. The 3 possible double-label combinations (Cx40 with Cx43, Cx37 with Cx40, and Cx37 with Cx43) are illustrated for the aorta in Figure 3. Precise localization of connexin label to the endothelium was made possible by using anti-VWF antibody as an endothelial marker (Figure 3A). Colocalization of 2 connexins within the same spot was readily detected as yellow fluorescence caused by direct superimposition of red and green colors. In aortic endothelium double labeled for Cx40 and Cx43 (Figure 3A), >85% of the spots positive for one of these connexins was also positive for the other connexin (Table). Double labeling for Cx40 and
Cx37 demonstrated that >80% of the Cx37-positive spots were also Cx40 positive (Figure 3B). On the other hand, as Cx40 labeling predominated over most of the endothelial area, <50% of the Cx40-positive spots shared Cx37 fluorescence. Similarly, in Cx37/Cx43 double-labeled samples, >70% of the Cx37-positive spots contained Cx43 signal (Figure 3C). A noticeable feature of Cx37 labeling apparent from these double-label combinations was its heterogeneous distribution. In contrast to the multiple connexin expression apparent in the endothelium, aortic smooth muscle expressed only 1 of the connexins investigated, Cx43 (Figure 3).

Whereas corresponding double-labeling experiments on pulmonary artery endothelium revealed a pattern similar to that of the aorta, the endothelia of intramural coronary arteries differed markedly by their lack of Cx43. These similarities and differences between the vessels are summarized in the Table and are strikingly apparent in triple-labeled arterial sections (Figure 4). Simultaneous visualization of Cx37, Cx40, and Cx43 in this way emphasizes the extensive colocalization in the endothelium of Cx37-positive spots with Cx40, seen as yellow fluorescence. Abundant Cx40 signal (without Cx37) is also seen in the endothelium, but Cx43 is confined to the surrounding cardiac muscle. Confirmation of this specific spatial distribution pattern by separate viewing of each connexin type is illustrated in Figure 5. This pattern was observed throughout intramural coronary arteries, extending down to arterioles (Figure 4, inset), although it should be noted that Cx43 does become detectable in the coronary artery endothelium close to its junction with the aorta.23

Triple labeling could only be carried out effectively on coronary artery as autofluorescence of elastic fibers interfered with visualization of FITC signal in the pulmonary artery and aorta. The specificity of all the findings illustrated was exhaustively and repeatedly confirmed in controls in which each primary antibody treatment was followed with each secondary antibody separately, in pairs, and with all 3 secondary antibodies.

Double Labeling of Connexins in En Face-Viewed Aortic Endothelium

To obtain a more comprehensive view of the distribution of the 3 connexin types, we developed a technique for confocal en face viewing of the endothelium in whole mounts of en bloc immunolabeled arterial rings. Success with this procedure was confined to the aorta, the strong elastic wall of which maintains a ring shape, protecting the luminal surface from damage during processing. Attempts with pulmonary artery failed owing to too thin a wall to support a patent lumen during processing, resulting in technically unsatisfactory staining and damage of the luminal surface. Coronary arteries were difficult to manipulate for the procedure because of their small size.

“Bird’s eye” views of the aortic luminal surface prepared using this approach revealed extensive expanses of endothelium; hundreds of consecutive sections would have been required to reconstruct equivalent areas. Moreover, the views obtained made it possible to examine connexin distribution in relation to the direction of the bloodstream. Single labeling for the individual connexins clearly demonstrated the abundance of Cx40 signal, which in projection views clearly delineated the endothelial cell borders (Figure 6). Although the size of each connexin spot varied, the level of signal intensity was similar from one area to the next. Cx43 immunolabeling was also widespread, although it was less extensive than Cx40. Cx43 signal varied in abundance from one area to the next, although <10% of cells were free of detectable Cx43 immunolabeling. For Cx37, screening of large expanses of the luminal surface revealed scattered patches of punctate labeling. Each patch of Cx37 labeling varied in size, consisting of tens to hundreds of cells, and was typically irregular in shape, taking the form of a distorted spindle in which the long axis lay parallel to the direction of the blood flow. Cx37 spots, although sharply defined, were typically smaller, less conspicuous, and less abundant than those for Cx40 and Cx43. In addition to this punctate labeling at cell borders typical of gap junctions, low-intensity diffuse Cx37 signal was frequently observed spread over zones at the end of the cell body (Figure 6), possibly representing cytoplasmic Cx37 protein. It should be emphasized that, while information on overall patterns of connexin distribution were obtainable by the en face technique, in the absence of data on the concentration and binding affinities of the primary and secondary antibodies used, differences in the relative amounts or intensities of immunoreactive signals do not necessarily directly reflect differences in the amounts of the 3 connexins investigated.

Analysis of Connexin Colocalization in Double-Label Experiments of Arterial Endothelia

<table>
<thead>
<tr>
<th>Assessed Artery</th>
<th>Connexins Assessed</th>
<th>37 +40</th>
<th>37+40</th>
<th>40+43</th>
<th>40+43</th>
<th>37+43</th>
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<td>82 ±4</td>
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<td>87 ±3</td>
<td>86 ±3</td>
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</tr>
<tr>
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<td>87 ±3</td>
<td>46 ±12</td>
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Shown are results from the different double-labeling experiments. The percentage given here involved taking a single connexin type (underlined) and determining how many spots for that connexin also contained the second connexin type examined in the double-label experiment.

A0 indicates aorta; PA, main pulmonary artery; and CA, coronary artery.

*P<0.01 between each of the connexin pairs in each artery type. No significant differences between the values for colocalization percentage were apparent within individual columns.
En face views of aortic endothelium after double labeling demonstrated just how extensive the colocalization was between Cx43 with Cx40 (Figure 7A) and Cx37 with Cx40 (Figure 7B). As with the sectional views, en face double labeling confirmed that the majority of Cx43-positive spots and the majority of Cx37-positive spots were in each case also positive for Cx40. For Cx37 and Cx43, although substantial numbers of the Cx37-positive spots colocalized with Cx43-positive spots, zones in which labeling for 1 or the other of the 2 connexins predominated were apparent (Figure 7C).

Apart from extending information on connexin distribution seen in sections, the en face technique permitted detailed assessment of the spatial patterns of colocalization circumscribing individual cells. The extent of colocalization varied both from 1 cell to the next and around the periphery of individual cells. One common pattern involved extensive colocalization of 2 connexins at the border of a given cell with one of its neighbors but less or no colocalization where the same cell abutted a different neighbor (Figure 7).

**Immunogold Electron Microscopy**
Given that, by means of the en face approach, the most comprehensive picture of connexin expression had been built up on rat aortic endothelium, we applied single and multiple immunogold labeling with electron microscopy to gain further information in this tissue. Single labeling of Lowicryl sections with the anti-Cx37 and the anti-Cx40 antibodies...
demonstrated specific binding to morphologically recognized gap junctions with virtually no labeling of other structures in the specimen (Figure 8). Single label controls in which the primary antibodies were omitted consistently revealed negligible gold labeling. Comparable results for the anti-Cx43 antibody used have been presented previously. These findings confirm that the fluorescent spots revealed in each case by immunoconfocal microscopy represent individual gap-junctional plaques. In the single-labeling experiments on Lowicryl sections, ≈100% of the gap junction profiles viewed were labeled with the Cx40 antibody, and ≈50% showed at least some labeling with the Cx37 antibody (observations based on >150 gap junctions from a minimum of 10 different sections for each connexin type). Double immunogold labeling demonstrated instances in which each pair of connexins could be successfully localized to gap junctions, as in the corresponding confocal experiments.

The key question raised by the immunoconfocal and immunoelectron double label experiments was whether all 3 connexin types occurred within single gap junctions. To address this question, triple labeling of Cx37, Cx40, and Cx43 was carried out by immunogold labeling of Lowicryl sections using gold markers of 3 distinct sizes. In these triple-label experiments, gap junctions with only 1 size of marker were seldom observed, and instances in which all 3 sizes of marker were localized to the same gap junction were consistently demonstrated (Figure 8). Gold markers for the multiple connexins were associated with both of the contributing plasma membranes of the gap junction (ie, the gold markers were not segregated across the junction, as would have been predicted if 1 cell produced 1 connexin, with its neighbor producing the other[s]). Specificity in the multiple-labeling experiments was demonstrated in controls in which sections were treated with all 3 secondary antibody-gold complexes after receiving (1) no primary antibody, (2) Cx37 primary only, (3) Cx40 primary only, or (4) Cx43 primary only (Figure 8D through 8G). In the absence of any primary antibody, no binding of any of the 3 secondary antibodies was observed (Figure 8D). After treatment with the Cx37 primary antibody, only the appropriate 5-nm secondary antibodies bound to gap junctions, and background labeling over nonjunctional areas for all 3 secondary antibodies was negligible (Figure 8E). Corresponding results were obtained after treatment with the Cx40 primary antibody and after treatment with the Cx43 primary antibody; the only secondary antibody to bind was that matching the primary antibody, such binding occurred specifically at gap junctions, and negligible gold label of any size was observable over other structures in the samples (Figure 8F and 8G).

Freeze-fracture cytochemistry extended and confirmed the results obtained on Lowicryl sections by permitting visual-
Integration of typical planar freeze-fracture replica views of gap junctions together with superimposed immunogold label (Figure 9). Triple labeling using 3 sizes of gold marker in these en face views demonstrated that Cx40, Cx43, and Cx37 were frequently simultaneously present in the same gap-junctional plaque. The most reliable assessment of the results of triple labeling came from inspection of E-face views of the junction; the sharply contrasting detail of connexons on P-face views interfered with discrimination of small-diameter gold label (E face is the fracture face of the half-membrane leaflet left attached to the extracellular space after freeze fracture; P face is the fracture face of the half-membrane leaflet left attached to the protoplasm). Negligible gold label was apparent in intervening areas of the membrane. The 3 labeled junctions illustrated in Figure 8 are typical of 150 gap junction plaques viewed after triple label/freeze-fracture cytochemistry. Controls, following the same principles as those applied to sections, confirmed specificity of labeling.

Discussion
The key findings of the present study are as follows: (1) multiple expression of connexins is a characteristic feature of individual endothelial cells from different arterial sites in the intact tissue, (2) up to 3 connexin types occur within individual endothelial gap-junctional plaques, and (3) the precise mix and match of connexin expression varies in different arterial segments and along the border of a given cell as it makes contact with different neighbors. These findings, which expand existing knowledge of the organization and connexin makeup of endothelial gap junctions, were the outcome of specifically developed multiple-localization strategies based on the use of anti-connexin antibodies of demonstrated specificity raised in different species, development of novel techniques for en face viewing of the endothelium by confocal microscopy, and application of multiple immunolabeling to permit simultaneous visualization of up to 3 connexin types for correlative immunoconfocal and immunoelectron microscopy.

While earlier studies established that Cx40 mRNA is expressed in vascular tissue15 and that the mRNAs encoding Cx37 and Cx43 were localized to endothelial cells,2,16,33 these studies examined the transcripts individually in diverse systems, and corresponding analysis of endothelial connexin expression at the protein level has similarly remained fragmentary.5,19,34–36 A major factor inhibiting progress has been the limited availability of suitable antibody probes; in particular, reliable Cx37 antibodies have proven difficult to produce.5 Of the 4 Cx37 antibodies previously reported,19,21,37,38 Western blot characterization has been given for only 2, and electron-microscopic immunogold characterization for none. This last form of characterization is critical, as it is the only method by which specific binding of the antibody to morphologically defined gap junctions, rather than some unrelated protein sharing the same epitope, can be definitively
Figure 7. A, En face confocal view of the endothelial pavement after en bloc double immunolabeling of rat aortic endothelium for Cx40 (green) and Cx43 (red). Each endothelial cell is clearly outlined by discontinuous beaded strands of punctate fluorescence label. The long axis of the endothelial cells is parallel to the direction of the blood flow. The numerous yellow spots mark sites of colocalization of the 2 connexins. Elsewhere, Cx40 predominates, although some Cx43 (red) spots are also seen. The heterogeneous distribution of yellow spots indicates that the extent of colocalization varies among cells and within individual cells. *Indicates a cell that has many spots positive for the 2 connexins at its right border but fewer such spots at its left border, where it abuts a different cell. Bar = 5 μm. B, En face confocal view of the endothelial pavement after en bloc double immunolabeling of rat aortic endothelium for Cx37 (red) and Cx40 (green). This image is taken from an area rich in Cx37 and oriented in a similar manner to Figure 6A. In Cx37-rich areas, the yellow punctate labeling indicates that Cx37 is colocalized with Cx40. Elsewhere, green fluorescence, indicating Cx40 in the absence of detectable Cx37, is seen. Virtually no bright punctate red signal is observed at the cell borders, showing that Cx37 seldom occurs in the absence of Cx40. However, patches of diffuse Cx37 signal are apparent in the cytoplasm of some cells that show punctate Cx37 label at their borders. *Indicates a cell that has many spots positive for the 2 connexins at the middle portion of its right border shared with another adjacent cell. Bar = 5 μm. C, En face confocal view of the endothelial pavement after en bloc double immunolabeling of rat aortic endothelium for Cx37 (red) and Cx43 (green). This image, oriented as in Figure 6A and 6B, is taken from an area where colocalization of Cx37 with Cx43 is low, to illustrate localized differential expression of these connexins by some groups of cells. The majority of spots are either red or green, indicating a relatively low extent of colocalization compared with the 2 combinations in the preceding figures. Fewer cells are outlined over their entire borders compared with those in Figure 6A and 6B, reflecting the lower quantities of Cx37 and Cx43 than Cx40. *Indicates a cell that expresses Cx37 on its left border, where it abuts other Cx37-expressing cells, but mainly expresses Cx43 on its right border, where it faces a patch of cells predominantly expressing Cx43. Note that in other areas, superimposition of Cx37 and Cx43 signal was observed. Bar = 5 μm.
established. The comprehensive characterization presented here for both our Cx37 and Cx40 antibodies, involving Western blots, transfected cells, and immunogold electron microscopy, is an essential foundation to reliably establishing connexin makeup at the level of the individual gap junction.

Although subject to the above limitations, recent studies using single immunofluorescence labeling have given suggestive evidence for the presence of connexins 43, 37, and/or 40 protein in the endothelium from specific vessels in situ; all 3 proteins were reported to be present in the endothelium of human umbilical vessels and rat aorta, while connexin 43 and 37 proteins (but not Cx40) were reported in porcine aortic endothelium. However, single labeling, even when conducted on serial sections, only hints at the extent to which multiple connexins are expressed by the same endothelial cell and, crucially, provides no information on which connexin combinations occur within individual gap-junctional plaques. Localization of 2 or more connexins to precisely the same subcellular site can only be definitively demonstrated by applying multiple labeling techniques to the same section. This was accomplished in the present study by generating and applying anti-connexin antibodies raised in 3 different species to enable use of specific secondary antibody detection systems (directed against each species), combined with application of up to 3 distinct fluorochromes for confocal microscopy and 3 sizes of gold marker for electron microscopy, thereby permitting simultaneous localization of the multiple connexin types.
It should be emphasized that colocalization of fluorescent signals for 2 connexin types at the confocal microscopic level, as has been reported in a number of nonendothelial systems (eg, epidermal cells, cardiomyocytes, ovarian granulosa cells, and ciliary body), suggests but does not constitute proof for the presence of 2 connexin types within the same gap-junctional plaque. Immunofluorescence localization strictly only permits detection of a protein; it does not actually visualize the gap junction itself, and confocal visualization of immunolabeled spots positive for 2 fluorochromes may in theory arise from 2 closely associated junctions. Complementary immunoelectron microscopy, as conducted here, is essential to permit visualization of the junction itself and hence interpret fully the immunoconfocal observations. Immunoelectron microscopy has previously demonstrated mixtures of 2 connexin types in gap junctions of nonvascular cells (eg, Cx32 and Cx26 in liver and Cx46 and Cx50 in lens), and we recently presented evidence for colocalization of Cx40 and Cx43 in endothelial gap junctions of rat aorta. The present study now extends this approach by applying triple immunogold labeling to demonstrate the simultaneous presence of 3 connexin types in the same gap-junctional plaque. Our double and triple connexin localization data clearly demonstrate that not only do the endothelia of different segments of the arterial tree (viz, coronary artery versus pulmonary artery and aorta) show characteristic differences in immunodetectable connexin
Multiple Connexins in Endothelial Gap Junctions

Cx37

A

Cx40

B

Cx37/Cx40/Cx43

C

Controls

D

E

F

G
expression patterns, but the multiplicity of connexin expression patterns extends to the level of the individual gap junction itself, with rat aortic endothelial gap junctions typically containing Cx43 or Cx37, in addition to Cx40, or all 3 connexin types.

Whether the expression of multiple connexins reflects biological redundancy or whether the different connexins play distinctive functional roles cannot be deduced from the morphological data presented. However, functional data from in vitro expression systems does at least raise the possibility, however speculative, that the presence of multiple connexins within individual gap junctions could potentially offer scope for modulation of intercellular communication properties in vivo. Analysis of in vitro expression systems indicates that the properties of gap junction channels, such as molecular permeability, ionic selectivity, unitary conductance, and voltage gating vary according to the specific connexin expressed.7,9–13 Cx37 channels, for example, typically have higher unitary conductance values (300 pS) than do Cx40 channels (150 to 200 pS) or Cx43 channels (60 pS).46–49 Furthermore, channel properties are altered when cell lines endogenously expressing 1 connexin type are transfected with a second connexin type50 and when cells are cotransfected to express 2 connexin types.51 Evidence that distinctive patterns of connexin expression contribute to modulation of function in the endothelium comes from recent studies in cultured bovine aortic endothelial cells showing that Cx43 and Cx37 are differentially regulated according to growth
status. In this system, Cx43 levels become elevated during growth but decline at confluency, while Cx37 transcript levels show the reverse pattern. Differences in Cx43 and Cx37 expression along the border of an individual cell where it abuts different neighbors, as observed in the present study, could conceivably reflect different stages in the growth cycle of adjacent cells within the endothelial monolayer in situ.

Apart from the specific type of connexin expressed, the idea that the precise arrangement of different connexins at the levels of the channel and junctional plaque may be key determinants of gap junction function is currently the focus of considerable speculation. In nonvascular tissues, evidence suggestive of heterotypic channels (ie, channels in which 1 connexon made from 1 connexin type abuts a partner connexon made from a second connexin type) and heteromeric connexons (containing mixtures of connexins within the connexon) has been presented. An alternative arrangement, in cells coexpressing 2 connexins, is that the individual connexins aggregate into separate gap junctions or are organized into different homotypic channel domains within the same junctional plaque. The simultaneous presence of up to 3 connexin types within endothelial gap junctions as demonstrated here evidently does not conform to the former pattern (ie, segregation of connexins into separate gap junctions) but is compatible with a variety of other arrangements of connexins at the level of the individual gap junction channel, including heterotypic channels, heteromeric connexons, or mixtures of different types of homotypic channels within individual gap junction plaques. This wide range of possible structural arrangements could open a correspondingly wide spectrum of possibilities for modulation of intercellular communication properties. In vitro expression systems, connexons composed of Cx43 do not form functional channels with those composed of Cx40, owing to inability to dock. However, Cx37 channels are compatible with both Cx43 channels and Cx40 channels. Furthermore, junctions formed between cell pairs cotransfected to express Cx43 and Cx37 form different channel types from those expressing only Cx43 or only Cx37 (ie, Cx43/Cx43 pairs, Cx37/Cx37 pairs, and Cx43/Cx37 pairs). These data raise the speculative possibility that Cx37 in endothelial cells may have the capacity to facilitate continuation of functional linkage in gap junctions containing the otherwise incompatible Cx43 and Cx40 and that, in addition to heterotypic Cx37/Cx40 and Cx37/Cx43 channels, formation of heteromeric Cx37/Cx43 channels with functional features distinct from the latter may be formed.

In conclusion, the present findings raise the possibility that the connexin makeup of aortic endothelial gap junctions, involving 3 connexin types conferring different properties in vitro, may provide inherent potential for complex regulation, functional differentiation, and versatility of endothelial intercellular communication properties in vivo. A clearer idea of the functional consequences of specific connexin combinations should emerge in the future with the development of in vitro systems manipulated to mirror the multiple connexin expression patterns observed in vivo, in combination with the accumulating data from knockout studies.

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