ARTICLE

Gap Junction Localization and Connexin Expression in Cytochemically Identified Endothelial Cells of Arterial Tissue

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

Imperial College School of Medicine at National Heart and Lung Institute, London, United Kingdom

SUMMARY  Vascular endothelial cells interact with one another via gap junctions, but information on the precise connexin make-up of endothelial gap junctions in intact arterial tissue is limited. One factor contributing to this lack of information is that standard immunocytochemical methodologies applied to arterial sections do not readily permit unequivocal localization of connexin immunolabeling to endothelium. Here we introduce a method for multiple labeling with specific endothelial cell markers and one or more connexin-specific antibodies which overcomes this limitation. Applying this method to localize connexins 43, 40, and 37 by confocal microscopy, we show that the three connexin types have quite distinctive labeling patterns in different vessels. Whereas endothelial cells of rat aorta and coronary artery characteristically show extensive, prominent connexin40, and heterogeneous scattered connexin37, the former, unlike the latter, also has abundant connexin43. The relative lack of connexin43 in coronary artery endothelium was confirmed in both rat and human using three alternative antibodies. In the aorta, connexins43 and 40 commonly co-localize to the same junctional plaque. Even within a given type of endothelium, zonal variation in connexin expression was apparent. In rat endocardium, a zone just below the mitral valve region is marked by expression of greater quantities of connexin43 than surrounding areas. These results are consistent with the idea that differential expression of connexins may contribute to modulation of endothelial gap junction function in different segments and subzones of the arterial system.

KEY WORDS  gap junctions, endothelium, connexins, artery, heart, rat, human, confocal microscopy

The vascular endothelium forms a continuous monolayer lining the luminal surface of the entire cardiovascular system, providing the structural and metabolic interface between the blood and underlying tissues. Endothelial integrity is essential for maintenance of healthy tissue function, and perturbations of endothelial structure and function are critical to the pathogenesis of vascular disease (Ross 1995; Stary et al. 1994). Integration of endothelial cell functions is mediated by a variety of intercellular signaling mechanisms, including direct cell-to-cell communication via gap junctions (Larson 1988). Gap junctions are specialized cell membrane domains consisting of clusters of protein channels that link the cytoplasmic compartments of neighboring cells, forming pathways for direct exchange of ions and small molecules (for reviews see Yamasaki and Naus 1996; Chanson and Spray 1995; Gourdie 1995; Wolburg and Rohlmann 1995; Beyer 1993; Gilula, 1992). The component proteins of gap junction channels are connexins, a multigene family of conserved proteins, different members of which are expressed in different cell types, tissues, and species (for reviews see Bruzzone et al. 1996; Kumar and Gilula 1996). At least 12 different mammalian connexins have thus far been identified, of which three, connexins37, 40, and 43, are expressed in the vascular wall (Beyer et al. 1992; Willecke et al. 1991; Larson et al. 1990). Whereas vascular smooth muscle cells predominantly express connexin43 and, in some instances, connexin40 (Blackburn et al. 1995; Little et al. 1995a; Moore and Burt 1994,1995; Rennick et al. 1993), en...

Correspondence to: Prof. N. J. Severs, Cardiac Medicine, Imperial College School of Medicine at National Heart and Lung Institute, Royal Brompton Hospital, Sydney Street, London SW3 6NP, UK.

Received for publication June 19, 1996; accepted November 14, 1996 (JH 4A 40 13).
dothelial cells are reported to express connexins 43, 40, and 37 (Blackburn et al. 1995; Little et al. 1995a; Reed et al. 1993; Pepper et al. 1992). In vitro studies have demonstrated that permeability, conductance, and other properties of gap junctional channels depend on the precise make-up of their component connexins (Bello et al. 1995; Elfgang et al. 1995; Little et al. 1995b; Veenstra et al. 1995). The possibility therefore exists that expression of different combinations of connexins may contribute to functional differentiation in different zones of the vascular system.

Information to date on the expression of connexins in endothelial cells has relied largely on the use of cultured cells and on Northern and Western blotting of endothelial scrapings. In situ immunocytochemical localization studies of the intact vessel have thus far been largely restricted to the microvasculature (e.g., Little et al. 1995a). Knowledge of the spatial distribution of endothelial connexins, especially in large arteries, therefore remains limited. One factor contributing to this dearth of information is the difficulty of unequivocally identifying endothelial cells in sections of the immunolabeled intact arterial wall. The fine, delicate nature of endothelial cells makes their discrimination by microscopy difficult in immunolabeled preparations and renders them liable to detachment during tissue preparation or pathological processes. Although endothelial cell cultures provide much useful information, and methods also exist for the stripping of endothelial cell monolayers for in vitro viewing with immunohistochemical staining (Schwartz and Benditt 1973), these approaches preclude the study of the neighboring subendothelial area, which is of key importance to the interactions involved in disease histogenesis and normal tissue function. Moreover, the connexin repertoire of cultured cells does not always replicate that of their counterparts in the intact tissue.

Investigation of connexin expression in the endothelium of the intact vessel therefore requires simultaneous visualization of unequivocally identified endothelial cells with immunolabeled gap junctions. To this end, the present study set out to develop a method for multiple labeling with specific endothelial cell markers and one or more connexin-specific antibodies. Application of this method has made it possible to initiate investigation of the distribution of gap junctions and the diversity of connexin expression in endothelial cells in the intact tissue in a range of arterial vessel types.

Materials and Methods

Tissue Preparation

Specimens of aorta, coronary artery, and endocardium were obtained from adult male Sprague-Dawley rats (318–450 g). One series of five animals was sacrificed by dislocation of the neck and the arterial samples were rapidly washed with PBS containing heparin (10 U/ml), cut into 5-mm-thick segments, and frozen immediately without fixation. A second series of six animals was perfusion-fixed in paraformaldehyde before freezing. These animals were anesthetized by ip injection of midazolam–hypnorm and perfused retrogradely, via a catheter in the abdominal aorta, with heparinized PBS, followed by phosphate-buffered 2% paraformaldehyde (pH 7.4) for 20 min. Five-mm-thick transverse rings of fixed arterial tissues were incubated in 30% sucrose in PBS for 1 hr before freezing. Preparation of rat tissues was conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986.

Human coronary arteries were also studied. Samples of undiseased coronary artery were obtained from the explanted hearts of five male patients (mean age 45.3 ± 6.7 years) with endstage dilated cardiomyopathy. After ischemic arrest and removal of the heart, segments of coronary artery were immediately dissected out in the operating theater and placed in 2% paraformaldehyde (phosphate-buffered) for 1 hr, followed by PBS wash. Up to nine segments from different coronary arteries were obtained per heart. From each segment, one 5-mm-thick transverse arterial ring was selected for study. Work on human tissues was conducted according to institutional ethical committee policies.

All samples were swiftly immersed in isopentane held close to its freezing point (−160°C) using liquid nitrogen, and subsequently stored under liquid nitrogen before cryosectioning. Ten-μm-thick cryosections of the arterial rings were cut transversely and obliquely using a cryostat. Oblique cutting provided extended tangential sections in which portions of endothelium were viewed en face.

Immunofluorescence Labeling of Gap Junctional Connexins and Endothelial Cell Identification

Anti-connexin Antibodies. Three principal antibodies were used for the immunofluorescence detection of the gap junctional proteins, connexins 37, 40, and 43. Those against connexins 37 and 40 were polyclonal antisera raised in rabbits. The connexin37 antiserum was raised against a synthetic peptide corresponding to residues 266–281 (“D37”) of the cytoplasmic C-terminal tail of the rat connexin37 (a gift from P. M eda) (M eda et al. 1995). The connexin40 antiserum was produced against a synthetic peptide corresponding to residues 254–268 of the cytoplasmic C-terminal tail of the rat connexin40 sequence. Both the connexin37 and connexin40 antisera were affinity-purified on the respective immobilized peptides. For connexin43, a mouse monoclonal antibody (against residues 252-270 of rat connexin43) (Beyer et al. 1987) was purchased from Chemicon (Harrow, UK). In addition, the results were verified using alternative antibodies to the same connexin types as follows: (a) for connexin 37, rabbit polyclonal antiserum raised against residues 244–255 (“R37”) of rat connexin37; (b) for connexin43, rabbit polyclonal antiserum raised against residues 131–142 (“H”) (Green and Severs 1993; Harfst et al. 1990) and residues 314–322 (“C16”) of the rat connexin43 sequence (El Aoumari et al. 1990).

Endothelial Cell Markers. Three endothelial cell markers were used, as follows. The lectin Ulex europaeus agglutinin...
1 (UEA1) conjugated with FITC, and Bandeiraea simplicifolia isoelectin B4 (BS1) conjugated with FITC, both purchased from Sigma (Dorset, UK), were used to label endothelial cells of human and rat arteries, respectively. As an alternative, rabbit anti-human Von Willebrand factor (anti-VWF) polyclonal antiserum (Dako; Wycombe, UK) was used to label endothelial cells of both rat and human specimens.

Secondary Antibody/Detection Systems. For standard connexin labeling, both in single labeling experiments and in combination with endothelial marking (see below), the secondary antibody/detection systems used were biotinylated sheep anti-rabbit or biotinylated sheep anti-mouse immunoglobulins with Texas Red–streptavidin (Amersham Life Science; Poole, UK). For double labeling of two connexin isoforms (both with and without endothelial marking), we used donkey anti-mouse immunoglobulin conjugated to CY5 and donkey anti-rabbit immunoglobulin conjugated to CY3 (both from Chemicon). Secondary antibody/detection systems used for endothelial marking were as follows: (a) rabbit anti-fluorescein isothiocyanate immunoglobulin (anti-FITC; Dako); (b) swine anti-rabbit–fluorescein isothiocyanate (anti-rabbit–FITC; Dako). Reagents a and b were used to amplify the signal associated with lectin. Reagent b alone was used for labeling of Von Willebrand factor.

Combined Labeling of Gap Junctions with Endothelial Marking. Sections were mounted on poly-l-lysine-coated slides. After overnight air-drying in the −20°C freezer, the unfixed rat sections were first immersed in −20°C methanol for 5 min, after which all sections were rinsed in PBS for 5 min and treated with 0.1% Triton X-100 in PBS for 15 min. This was followed by blocking in PBS containing 0.5% bovine serum albumin for 15 min and incubation with the anti-connexin antibody of choice. Different conditions were found to be optimal for each of the antibodies, as follows: connexin37 antibody (dilution 1:300) at room temperature (RT) overnight; connexin40 antibody (dilution 1:1000) at 37°C for 30 min; connexin43 monoclonal antibody (dilution 1:1000) at RT overnight. Sections were then treated for 1 hr at RT with biotinylated sheep anti-rabbit (for connexins 37 and 40) or biotinylated sheep anti-mouse (for connexin 43) at a concentration of 1:250, with subsequent fluorescent visualization using Texas Red–streptavidin (1:250). In experiments in which two connexins were simultaneously localized in the same section, sequential incubation with each of the anti-connexin antibodies was followed by incubation at RT for 1 hr with a mixture of the two secondary antibodies (CY3 and CY5; 1:250). This approach was feasible for the combination of connexins 40 and 43.

For simultaneous marking of endothelial cells, one of two alternative procedures was used: lectin labeling or localization of VWF. For the former, the connexin immunolabeled sections were exposed either to lectin BS1–FITC (5 μg/ml in PBS) at RT for 2 hr (for rat specimens) or lectin UEA1–FITC (1 μg/ml in PBS) for 1 hr (for human specimens). These sections were then incubated for 1 hr at RT in rabbit anti-FITC (dilution 1:500), followed by swine anti-rabbit–FITC (dilution 1:25) for 1 hr at RT to enhance fluorescent visualization. For the VWF method, the sections were treated for 1 hr at RT with anti-VWF (dilution 1:500), followed by swine anti-rabbit–FITC (dilution 1:25). After washing in PBS, the slides were mounted with Citifluor mounting medium (Agar; Essex, UK). The sections were given a thorough wash in PBS between each step. The endothelial marking approach was successfully applied to sections that had been prelabeled for one or for two connexin types.

Corresponding experiments were conducted in which each of the anti-connexin antibodies was applied alone (i.e., no endothelial marker) and the endothelial marker used alone (i.e., no connexin labeling). As positive controls, gap junction labeling in the medial smooth muscle cells (connexin 43 antibody) and in the endothelial cells of vasa vasorum (connexin 40 and 37 antisera) was used. Negative controls included omission of primary antibody and peptide inhibition. In addition, each secondary reagent was confirmed to be species-specific by secondary antibody crossover (i.e., mouse primary antibody followed by anti-rabbit secondary; rabbit primary antibody followed by anti-mouse secondary).

Confocal Laser Scanning Microscopy and Correlative Histology

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 detection of fluorescein, Texas Red, and CY3 and CY5 fluorescence. The images were taken using simultaneous dual or triple channel scanning and transformed into projection views using sets of five consecutive single optical sections taken at 1-μm intervals. All specimens were examined within 24 hr of immunolabeling. Adjacent sections to those used for immunolabeling, stained with hematoxylin and eosin, were examined using standard brightfield optics for comparative histological examination.

<table>
<thead>
<tr>
<th>Connexin isoform</th>
<th>Rat aorta</th>
<th>Rat endocardium</th>
<th>Rat coronary artery</th>
<th>Human coronary artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>Heterogeneous</td>
<td>Heterogeneous</td>
<td>Heterogeneous</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td></td>
<td>+ to ++++</td>
<td>+ to ++++</td>
<td>+ to ++++</td>
<td>+ to ++++</td>
</tr>
<tr>
<td>40</td>
<td>Uniform</td>
<td>Uniform</td>
<td>Uniform</td>
<td>Uniform</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>+ to ++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>43</td>
<td>Uniform</td>
<td>Heterogeneous</td>
<td>Sparse</td>
<td>Sparse</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>+ to ++++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Semiquantification by scoring + to ++++ to indicate percentage of marked endothelial area which included distinct punctate connexin immunolabeling delineating the cell borders, as follows: +, <1%; +, 1–25%; ++, 26–50%; +++, 51–75%; ++++, 76–100%. Note that these percentages reflect relative differences in the extent of labeling. They do not represent the absolute percentage of endothelial area occupied by gap junctions.
For comparison of the distribution and relative quantities of the immunofluorescence signal for each connexin type in the different vessels, 10 sections were taken from each of six samples for each rat arterial type or, for human coronary artery, from five separate samples. Each sample came from a different animal or patient. The sections were assessed visually at a magnification of ×400 for the distribution of connexin immunofluorescence and were scored for the relative amount of signal by estimating the percentage of endothelial area (as revealed with the cell marker) that exhibited distinct punctate connexin labeling delineating the cell borders. With this system, the maximum score of 100%/+ + + + + + + was applied where punctate label was apparent around the borders of all endothelial cells observed, and the minimal score of <1% to instances in which less than 1% of the endothelial area observed included positive connexin signal at the cell borders. Intermediate scores between these extremes are as defined in the footnote to Table 1. It should be noted that differences in signal intensity in immunolabeling experiments do not necessarily directly reflect relative abundance of different connexin types. Other factors relating to the distinctive properties of different antibodies (e.g., affinity for their respective antigens, sensitivity to processing protocols) are also involved. In the present study, however, the distribution patterns and extent of labeling for connexin37 were reproducibly observed with two separate antibodies and those for connexin43 with three separate antibodies.

**Postembedding Immunogold Labeling for Electron Microscopy**

To verify results obtained from double labeling for connexins 40 and 43 at the electron microscopic level, we carried out immunogold labeling of sections of rat aorta that had been embedded at low temperature in Lowicryl K4M (Carlemalm et al. 1981). Rats were perfusion-fixed with 2% paraformaldehyde in PBS (pH 7.4) for 15 min and the aortic arch dissected out. Fixation was continued in the same solution for a further 15 min at RT. The samples were then dehydrated in 30% ethanol at 4°C for 30 min, followed by 50% and 70% ethanol at −20°C for 30 min and 1 hr, respectively, and then 90% and absolute ethanol (three times) at −30°C with 1 hr for each step. Infiltration was at −30°C for periods of 1 hr with

**Figures 2-4** Combined endothelial cell marking and connexin43 labeling in rat aortic endothelium. The left column of figures (A) shows connexin43 labeling without endothelial marking; the right column of figures (B) shows identical fields with endothelial marker. Connexin43 gap junctions (red fluorescent spots) occur in the endothelium (green fluorescence in right column), and in the underlying medial smooth muscle cells (m). **Figures 2 and 3** show different aspects of tangentially sectioned endothelium (Plane A in Figure 1). In Figure 2, the position of the endothelium is suggested from the pattern and abundance of connexin43 at the luminal surface. In Figure 3, however, the position of the endothelium can be determined only by using the marker. **Figure 4** illustrates transversely sectioned endothelium (Plane B in Figure 1). The endothelial marker in these examples is VWF; connexin43 gap junctions were detected using mouse monoclonal antibody. Bars = 25 μm.

**Figure 5** Connexin40 labeling (red fluorescent spots) in rat aortic endothelium (fluorescent green marker). Connexin40 gap junctions are abundant specifically in the endothelium; no immunolabeling is detectable in the medial smooth muscle. (A) Endothelium that has been detached from the vessel wall, a factor that, in the absence of endothelial marking, could lead to misinterpretation. (B) An example in which the endothelium has remained attached. The endothelial cell marker in A was BS1 lectin; that in B was VWF. Bar = 25 μm.

**Figure 6** Connexin37 labeling (red fluorescent spots) in rat aortic endothelium (fluorescent green marker). Connexin37 immunolabeling occurs as heterogeneously distributed small spots (A) but is absent from major stretches of the endothelium (B). Endothelial marker, VWF; connexin37 detected with “D37.” Bar = 25 μm.
1:1 and 2:1 mixtures of Lowicryl K4M:ethanol, followed by pure Lowicryl (Agar Scientific; Stansted, UK) overnight. After a further wash in pure resin for 1 hr, the samples were then embedded in fresh pure resin in gelatin capsules, and polymerized with uv light at −30°C for 16 hr and then at RT for up to 72 hr in a Balzers FSU 010 low temperature embedding unit. Ultrathin sections were processed for single and double labeling using connexin43 and connexin40 primary antibodies (diluted 1:500) in PBS containing 0.5% BSA, followed by incubation in a mixture of 10-nm gold/goat anti-rabbit complexes and 5-nm gold/goat anti-mouse complexes (BioCell; Cardiff, UK) diluted 1:50 in PBS for 1 hr, following standard procedures (Slot and Geuze 1984). Negative controls consisted of omission of primary reagents. Both secondary reagents were confirmed to be specific to their appropriate primary antibody. All sections were examined in the Philips EM 301 electron microscope.

Results

In arterial sections conventionally stained with hematoxylin and eosin, endothelial cells are typically viewed in a variety of orientations, ranging from transverse to oblique (Figure 1). In sections immunolabeled with anti-connexin antibodies, the limits of the endothelium could not always be discerned with confidence, even with optimized correlative phase-contrast examination and, consequently, attributing positive or negative signal at the luminal surface to endothelium was problematic. Figures 2–5, taken from rat aorta, illustrate this problem and how it can be effectively overcome using endothelial cell markers.

Figures 2A and 3A illustrate typical patterns of connexin43 labeling observed in the absence of endothelial marker. In the former the labeling increases in abundance in a band towards the luminal side, and in the latter uniform labeling is observed extending to the luminal edge. By simultaneously marking the endothelium with anti-VWF (Figure 2B), it becomes clear that the band of increased labeling in Figure 2A coincides precisely with the presence of tangentially cut endothelium (as depicted in Figure 1A). The absence of a similar band of elevated connexin43 labeling in Figure 3A might be attributed to loss of the endothelium. However, application of the endothelial marker demonstrates a narrower band of tangentially cut endothelium, which in this orientation reveals connexin43 gap junctions at a density similar to those of the underlying smooth muscle cells (Figure 3B). A comparable connexin43 gap junction pattern is apparent where the endothelium is viewed in precise transverse section (Figure 4), equivalent to the plane shown in Figure 1B.

By applying antibodies against other connexin types, we demonstrated that, apart from connexin43 (Figures 2–4), rat aortic endothelial cells express connexins 40 (Figure 5) and 37 (Figure 6). The examples in Figure 5 further illustrate how detachment of the endothelium during processing may, in the absence of endothelial marking, confound interpretation of connexin immunolabeling results, and that Bandeiraea simplicifolia isolectin B4 (Figure 5A) produces comparable results to anti-VWF (Figure 5B) when used as the endothelial marker. Comparison of the results obtained with antibodies against the three different connexin types revealed distinctive immunolabeling patterns in rat aortic endothelial cells. Whereas connexin43 signal was abundant in both the endothelium and medial smooth muscle cells, connexin40 was confined to the endothelium (Figures 2–5). Both connexin43 and connexin40 labeling revealed prominent punctate patterns delineating individual endothelial cells in en face views. Overall, the extent and intensity of connexin40 signal exceeded those of connexin43. Connexin37 dif-

---

**Figure 7** Dual labeling for connexin40 and 43 in the rat ventricular septum. The endocardial endothelium (blue marker) contains connexin43 gap junctions (red, indicated by arrow) but no connexin40 (green fluorescence). However, connexin40 is abundant in the myocytes of the left bundle branch (LBB) of the atrioventricular conduction system. The prominent labeling here results from superimposition of gap junctions over the depth of the section series. Beneath the bundle branch myocytes, typical patterns of connexin43 labeling are observed in intercalated disks of working ventricular myocardium (WV). Endothelial marker, VWF; connexin43 detected with mouse monoclonal antibody. Bar = 25 μm.

**Figure 8** Double labeling for connexin40 and 43 in rat coronary artery endothelial cells (blue marker) and surrounding myocardium. Prominent connexin40 labeling (red) is present in the endothelium. Connexin43 (green label) is absent from the endothelium but abundant in the surrounding working myocardium. Small blue streaks distant from the artery represent endothelial cells of the microvasculature. Endothelial marker, VWF; connexin43 detected with mouse monoclonal antibody. Bar = 50 μm.

**Figure 9** Connexin labeling in human coronary artery endothelium. The connexin labeling is shown as red label and the endothelial marker green. Connexin40 (A) is common, connexin37 (B) is found only in low amounts, and connexin43 (C) is virtually undetectable in the endothelium although present (arrows) in the underlying medial smooth muscle (m). Endothelial marker, UEA1 lectin; connexin37, “D37”; connexin43, mouse monoclonal antibody. Bar = 25 μm.

**Figure 10** Double labeling for connexin40 (green) and connexin43 (red) in rat aorta. In many instances (yellow fluorescence, arrow), the connexins localize to the same gap junctional plaque. Many gap junctions are viewed as green fluorescent spots, indicating connexin40 as the predominant component. A few endothelial gap junctions are seen as red (connexin43) fluorescence. Red spots in the media (m) represent connexin43 gap junctions between smooth muscle cells. Connexin43, mouse monoclonal antibody. Bar = 25 μm.
ferred from connexins 43 and 40 by showing a mark- edly heterogeneous distribution (Figure 6). Although focal areas of rat aortic endothelium revealed small but clearly resolved connexin37 label at the cells' borders (Figure 6A), major stretches of the neighboring endothelium revealed no connexin37 label (Figure 6B).

Comparison of different vessels revealed characteristic and distinctive patterns of endothelial connexin expression in each. For example, the endothelium of the rat ventricular endocardium just below the mitral valve region expressed only connexin43 (Figure 7), whereas elsewhere in the ventricular endocardium some connexin40 and 37 was also detectable, albeit in small quantities. Connexins40 and 37 were widespread in the atrial endocardial endothelium. In rat coronary arteries, connexin40 was expressed abundantly (Figure 8) whereas connexin43 was typically undetectable except in a zone close to its junction with the aorta. Connexin37 was present in the coronary arteries but showed a heterogeneous pattern of distribution similar to that of the aorta (not illustrated). For all vessels, the extent and intensity of the labeling patterns ob-

**Figure 11**  Double immunogold localization of connexin40 (10-nm gold) and connexin43 (5-nm gold) in a section of Lowicryl-embedded rat aorta. In the endothelium, both sizes of gold marker are found specifically at the gap junction (gj), demonstrating that a population of rat aortic endothelial gap junctions contain both connexin types, as suggested from the confocal image in Figure 10. Both sizes of gold marker are closely associated with each of the two membranes making up the junction. For each size of gold marker, the amount of label associated with each junctional membrane was similar, indicating the presence of both connexins in both junctional membranes. (Inset) High-magnification view of the double-labeled gap junction. L, lumen. Connexin43 detected with mouse monoclonal antibody. Bar = 250 nm.

**Figure 12**  Gap junctions (gj) between smooth muscle cells in the subjacent media of the same double-labeled preparations as that in Figure 11 are labeled only with 5-nm gold, indicating that the smooth muscle cells contain connexin43 but no detectable connexin40. (Inset) High-magnification view. ECM, extracellular matrix. Bar = 250 nm.
served for connexins 37 and 43 were identical when alternative antibodies were applied. Table 1 summarizes the connexin distribution characteristics for the vessels examined.

To examine whether the differences observed were a vessel-specific or species-specific feature, the pattern of connexins in rat coronary artery was compared with that in human coronary artery. In human coronary arterial endothelium, as in the rat, connexin40 was the most abundant connexin (Figure 9A) and connexin37 was heterogeneously distributed (Figure 9B). Connexin43 was virtually absent, although close inspection revealed very occasional small spots (Figure 9C). Histological assessment confirmed absence of atherosclerotic lesions in the human coronary artery specimens examined.

To determine the relationships between the distributions of two connexin types, experiments were conducted in which connexins 43 and 40 were simultaneously localized in cytochemically identified endothelial cells (Figures 7 and 8). Application of this technique to the endocardial endothelium and to the coronary endothelium emphasized differential expression of connexins in closely adjacent compartments comprising different cell types. In the endocardium, the connexin43-expressing endothelial cells were seen adjacent to myocytes of the left bundle branch of the atrioventricular conduction system which, as reported previously (Bastide et al. 1993; Gourdie et al. 1993; Kanter et al. 1993), predominantly express connexin40 (Figure 7). In the coronary artery, connexin40-expressing endothelial cells were seen adjacent to connexin43-expressing working myocytes (Figure 8). This approach, apart from delineating distinct communication compartments comprising different cell types, also provides information on the connexin make-up of gap junctional plaques within the endothelium. In the rat aorta, double labeling for confocal microscopy indicated co-localization of signal for connexins 40 and 43 in >75% of the endothelial gap junctional plaques (Figure 10). Most of the remaining gap junction population appeared to contain only connexin 40, although the presence of some connexin 43 in these junctions, below the limits of immunofluorescence detection, cannot be excluded. On double immunogold electron microscopy, >90% of aortic endothelial gap junction profiles examined showed labeling for both connexins 40 and 43 associated with each of the contributing plasma membranes (Figure 11). The gap junctions between underlying medial smooth muscle cells consistently contained only connexin 43 (Figure 12).

Discussion

This study has demonstrated the feasibility of simultaneously labeling endothelial cells with cell-specific markers and localizing one or more connexins in sections of the intact arterial wall. Such a strategy facilitates localizing precisely where different connexins are expressed and how gap junctions of different connexin content are organized in the arterial endothelium in situ.

Our comparison of connexin immunolabeling results with and without simultaneous visualization of the endothelium demonstrates the variety of interpretative difficulties that arise in the absence of cell marking. These difficulties stem from two principal features of the endothelium. First, endothelial cells in the normal artery are thin and flat, forming an inconspicuous monolayer along the inner interface of the vascular wall. Second, the endothelium is readily detached from the vascular wall during the course of processing and cryosectioning, and endothelial denudation is a common feature of vascular pathologies. Therefore, positive connexin labeling along the luminal interface does not necessarily represent that of endothelial cells. Conversely, lack of immunolabeling for a given connexin at the luminal surface may be due either to absence of the connexin or to absence of endothelial cells. Portions of endothelium are frequently tangentially sectioned, even in transversely cut arterial rings, so that the boundary between endothelium and the underlying medial layer in sectional views varies considerably in depth. Furthermore, because two of the connexins reported to be expressed in endothelial cells, connexin 43 and connexin 40, have also been reported in vascular smooth muscle cells (Beyer et al. 1992; Pepper et al. 1992; Blackburn et al. 1995; Little et al. 1995a; Moore and Burt 1994, 1995), the two cell types cannot be reliably distinguished on the basis of connexin immunolabeling patterns alone. All the interpretative uncertainties arising from these sources are overcome by our dual cell marking/connexin labeling technique, in which use of either a specific lectin or labeling of the characteristic endothelial product VWF (Wharton et al. 1990; Sehested and Hou-Jensen 1981), permits unequivocal correlation of connexin labeling with the endothelial layer.

The basic strategy of using two fluorochromes that emit different wavelength spectra [e.g., one green (FITC) with one red fluorochrome (e.g., Texas Red)], applied here to achieve simultaneous endothelial marking and connexin localization, is widely used in cytochemistry. Extending this principle, we used up to three fluorochromes in the present study in the following combinations: (a) FITC (endothelium) plus Texas Red (connexin); (b) FITC (endothelium) plus CY 3 and CY 5 (two distinct connexins). In the vascular wall, elastic laminae give strong autofluorescence, which tends to mask FITC fluorescence, and for this reason FITC labeling was unsuitable for labeling gap junctions in the vessel wall. Even when used as the endo-

Pulmonary artery and aorta) from both Northern blot and cells derived from a variety of vessel types and species been reported to be expressed in cultured endothelial studies in several significant respects. Connexin43 has result we report here differ from and extend previous earlier observations (Little et al. 1995a; Reed et al. 1993; Pepper et al. 1992), the immunolocalization re-

aver endothelial cells of different vessels is in keeping with connexins 43, 40, and 37 are expressed in arterial en-

sults we report here differ from and extend previous sendary study had the advantage of being sufficient-

ly strong without amplification. Because of its simplic-

ty, the VWF marker is our method of choice and should be applicable to endothelia of most vessels. How-

ever, because the quantity of VWF expressed in the endothelia of different vessels is reported to vary (Gebrane–Younès et al. 1991; Stephenson et al. 1986; M ukai et al. 1980), the equally effective although more time-consuming lectin approach may have useful application in those vessels in which the endothelial VWF concentration is low.

Earlier ultrastructural studies, especially those applying freeze-fracture electron microscopy, have pro-

vided a comprehensive picture of the organization, size, and distribution of endothelial gap junctions in situ (Severs 1989; Hüttner 1985; Schneeberger, 1981; Hüttner and Peters 1978; Simionescu et al. 1975, 1976). These studies have consistently shown that morphological features of gap junctions and tight junctions in the endothelium are not uniform but vary in type and form in different segments of the vasculature. Such segmental differentiation is not surprising in the context of the diverse functions of endothelium in different locations (Simionescu and Simionescu 1988).

The present observations on the overall distribution and abundance of gap junctions in aortic and coronary endothelium, as detected by anti-connexin antibodies and confocal microscopy, are consistent with the morphological characteristics of gap junctions as previously determined in these arteries by freeze-fracture (Severs 1989; Hüttner 1985; Schneeberger 1981; Hüttner and Peters 1978; Simionescu et al. 1975, 1976).

Although the overall morphological characteristics of endothelial gap junctions are well documented, knowledge of the precise connexin makeup of endothelial gap junctions and their in situ distribution in different arteries is still fragmentary. Although our finding that connexins 43, 40, and 37 are expressed in arterial endothelial cells of different vessels is in keeping with earlier observations (Little et al. 1995a; Reed et al. 1993; Pepper et al. 1992), the immunolocalization results we report here differ from and extend previous studies in several significant respects. Connexin43 has been reported to be expressed in cultured endothelial cells derived from a variety of vessel types and species (e.g., brain microvascular system, umbilical vein, pulmonary artery and aorta) from both Northern blot and immunofluorescence studies (Carter et al. 1996; Xie and Hu 1994; Pepper et al. 1992; Larson et al. 1990).

However, although connexin43 transcripts have been detected in freshly isolated bovine aortic endothelial cells (Larson et al. 1990), immunolabeling of intact rat aorta was reportedly to reveal connexin43 expression only in the medial smooth muscle layer (i.e., not in endothelium) (Bruzzone et al. 1993). The present study now provides clear evidence that connexin43 protein is expressed in endothelial cells of the intact rat aorta and, moreover, is one of the major connexins at this site. Although connexin40 was not detectable in a recent study on isolated and cultured porcine aortic endothelial cells (Carter et al. 1996), our detection of prominent connexin40 labeling in aortic and coronary artery endothelium is consistent with earlier reports in the intact vessel (Bastide et al. 1993; Bruzzone et al. 1993). However, in contrast to Bastide et al. (1993), we found that connexin40 was detectable in low quantities in the ventricular endocardium and in substantial quantities in the atrial endocardium. Carter et al. (1996) report diffuse intracellular immunolabeling for connexin37 in cultured endothelial cells, but information on connexin37 expression in endothelium has otherwise been restricted to detection of mRNA transcripts (Reed et al. 1993). The present study extends these data by demonstrating expression of connexin37 protein in the form of a heterogeneously distributed, clear punctate pattern.

An important finding to emerge from the present study is that complementary investigation of the three connexin types reveals quite distinctive labeling patterns in different vessels. For example, whereas aortic endothelial cells have extensive, prominent connexin40 and 43 with heterogeneously distributed connexin37 labeling, coronary artery endothelium, although showing similar features for connexin40 and 37, reveals a lack of connexin43 labeling. The reproducibility of these labeling patterns with alternative probes to the same connexin type suggests that these distinctive features reflect true underlying differences in the relative abundance of the three connexins, rather than being due to technical factors such as differences in antibody affinities or epitope accessibilities. The presence of three connexin types with distinct immunolabeling patterns in endothelium of different arteries has potentially important functional implications. Recent experimental studies on cells stably transfected with cDNAs encoding different connexins indicates that the properties of gap junction channels (e.g., unitary conductance, voltage sensitivity, molecular permeability, and ionic selectivity) vary according to the specific connexin expressed (Beblo et al. 1995; Elfgang et al. 1995; Little et al. 1995b; Veenstra et al. 1995; M oreno et al. 1994). In several (non-endothelial) cell types, the presence of more than one connexin has been documented within a given gap junctional plaque (Darrow et al. 1995; Little et al. 1995a; Sosinsky 1995; Gros et al.
Connexin Localization in Arterial Endothelium

1994), raising the possibility that formation of heterotypic channels (comprising one connexon composed of one connexin type joined to a second connexon composed of a second connexin type) or heteromeric connexons (containing mixtures of connexins within a connexon) may contribute to the modulation of gap junction properties.

Our experiments on simultaneous localization of connexins40 and 43 by immunofluorescent and immunoelectron microscopy indicate that, in rat aortic endothelium, although a few junctions may contain one or other of these connexins, the major population of junctions contains a mixture of the two. A similar co-localization has recently been reported by confocal microscopy in microvascular (arteriolar) endothelium from hamster cheek pouch (Little et al. 1995a). Experiments using in vitro expression systems indicate that although many combinations of heterotypic connexins form functional permeable channels, those formed from connexin40 and connexin43 are non-functional. Heterotypic channels formed from either one of these connexins with connexin37 do form functional channels (Elfgang et al. 1995; White et al. 1995). Among questions this raises for future work are whether, in aortic endothelial cells in vivo, connexin37 is involved in forming heterotypic channels with these two connexins; whether homotypic or heterotypic connexin40 and connexin43 channels are formed; and whether connexins 40 and 43 self-aggregate to form minidomains within the same gap junctional plaque [as reported for connexins32 and 26 in rodent liver (Sosinsky 1995)]. Although the electron microscopic immunogold results presented here exclude a heterotypic connexin43/40 pattern in which one cell expresses only connexin43 and its partner exclusively connexin40, the precise organization of connexins within such junctions requires further study.

In conclusion, the present findings demonstrate that segmental differentiation of vascular endothelium extends to differences in the distribution patterns and relative abundances of the specific types of gap junctional connexins expressed. Such differential expression of connexins may contribute to modulation of gap junction function in different segments of the arterial wall. The combined endothelial cell marking/connexin labeling approach for confocal microscopy introduced here will enable further detailed, reliable investigation of the diversity of endothelial connexin expression.

Acknowledgments

Supported in part by project grants from the British Heart Foundation (grant no. PG 93136) and the Wellcome Trust (grant no. 046218/Z/95).

We wish to thank all colleagues who contributed gifts of antibodies or participated in their production, in particular Dr Colin Green and Dr Robert Gourdie (anti-connexin43 “HJ” and anti-connexin40). The anti-connexin37 antibody used here was produced at the University of Geneva Medical School, and we thank Prof Paolo Meda and Dr J-A. Haefliger (DMIB, Lausanne) for this gift.

Literature Cited


