Connexin43 Is Highly Localized to Sites of Disturbed Flow in Rat Aortic Endothelium but Connexin37 and Connexin40 Are More Uniformly Distributed

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Abstract—Vascular endothelial cells are linked by gap junctions, which facilitate the propagation of electrical and chemical signals along the vessel wall. The aim of this study was to determine the distribution and identity of the gap junction structural proteins (connexins) expressed by endothelial cells in situ. Connexin expression in different regions of the rat aortic endothelium was analyzed with the use of indirect immunofluorescence microscopy and Western blotting. Connexin40 and connexin37 were present in most, if not all, of the thoracic and abdominal aortic endothelia in the form of maculae at cell-cell appositions. In contrast, connexin43 was undetectable in most endothelia but extremely abundant in small numbers of cells localized at the downstream edge of the ostia of branching vessels and at flow dividers, regions that experience turbulent shear stress from disturbed blood flow. To examine the relationship of shear stress and connexin43 expression, localized stress was induced by surgical coarctation of the aorta, which was sufficient to cause striking local upregulation of connexin43 within 8 days. Thus, increases in connexin43 levels are an endothelial response to mechanical stress. (Circ Res. 1998;83:636-643.)

Key Words: connexin ■ endothelium ■ circulation ■ aorta ■ gap junction

The vascular endothelium provides a continuous non-thrombogenic lining for blood vessels and represents the principal barrier to the passage of circulating blood cells and macromolecules from the bloodstream to the underlying interstitium. Intercellular contact between endothelial cells is an important aspect of their function and is accompanied by the establishment of several types of intercellular junctions. In particular, gap junctions, which contain intercellular channels allowing the direct movement of low-molecular-weight substances between adjacent cells, are frequently observed between endothelial cells and between endothelia and underlying smooth muscle cells.

Gap junctions are present throughout the vascular tree, although considerable variations in size and abundance are reported.1,2 Although the precise function of intercellular communication in the vascular wall has not been defined, several roles have been suggested. The propagation of vaso-motor responses along arterioles is most likely accomplished by communication of changes in membrane potential through gap junction channels coupling smooth muscle, endothelium, or both.3,4 Similarly, communication between endothelium and underlying smooth muscle may influence vascular tone in larger vessels.5,6 In addition, gap junctional coupling has been implicated in the control of endothelial cell migration and proliferation after injury to the intimal surface1 and during the de novo growth of blood vessels.7

There are conflicting reports relating to the identity and distribution of gap junction structural proteins (connexins) in the vascular wall. At least 13 different connexins have been identified in rodents by DNA cloning,8 and most are known to form channels with distinct physiological properties. In cultured bovine aortic endothelial cells, connexin43 (Cx43) mRNA and protein are abundant.2 In these cultures, Cx43 protein was observed as discrete maculae at cell-cell appositional areas, a distribution highly consistent with gap junctional plaques. In vivo, Yeh et al9 reported high levels of Cx43 in rat aorta, although they failed to detect it in coronary arteries. In apparent contrast, an in vivo study by Bruzzone et al10 identified Cx43-containing maculae in smooth muscle cells but not in the endothelium of rat aorta, which, instead, contained high levels of a different connexin, connexin40 (Cx40). In addition, it was suggested that a third connexin, connexin37 (Cx37), could be a component of junctions in the vascular wall on the basis of mRNA distribution.11-13 This was confirmed by immunocytochemical localization in the developing mouse heart.9,14,15 Thus far, mRNAs for other connexins have not been consistently detected in highly vascularized tissues.

To further investigate the discrepancies between previous studies, we mapped the regional distribution of Cx37, Cx40, and Cx43 in rodent aortic endothelium by immunofluorescence microscopy on en face preparations of the vessel wall. Cx40 was the most abundant connexin and was present at cell-cell appositions between most endothelia. Cx37 distribution was similar to Cx40 but was present in fewer puncta. In...
contrast, Cx43 expression was largely restricted to specific regions near the ostia of aortic branches, regions where blood flow becomes nonlaminar and endothelial cells are subjected to shear stress. To examine the relationship between shear stress and Cx43 expression, a flow disturbance was induced in a segment of abdominal aorta by coarctation. Within 8 days, a strong but local upregulation of Cx43 was observed at the leading edge of the coarct. On this basis, we conclude that Cx43 expression can be rapidly modulated by hemodynamic forces in vivo.

Materials and Methods

Antibody Preparation
Antibodies specific for Cx37 and Cx40 were raised in rabbits against purified fusion proteins. Portions containing amino acids 229 to 333 (Cx37) or amino acids 231 to 331 (Cx40) were fused to the carboxyl terminus of the Schistosoma japonicum glutathione S-transferase protein (GST). A 2-step procedure was used for purification of the antibodies. First, antibodies against GST and bacterial proteins in the sera were adsorbed to total proteins from the lysate of bacteria expressing GST coupled to Sepharose-4B. Unbound material was then adsorbed with purified fusion proteins for either Cx40 or Cx37 coupled to Sepharose-4B. Specific antibodies were eluted from each column with 0.1 mol/L glycine, pH 2.5. Antisera against Cx43 were affinity-purified with the use of a synthetic peptide as previously described. The specificity of the anti-Cx40 antibody was confirmed by probing Western blots containing lysates of Xenopus oocytes injected with cRNAs for Cx37, Cx40, and Cx43. Specific labeling was observed only in lanes containing Cx40 (data not shown). Cx37 antibodies were characterized previously in a similar fashion. A mouse monoclonal antibody produced against the same peptide used by Beyer et al was purchased from Zymed. Because of the low titer of specific antibody in the ascites fluid provided, the monoclonal antibody was affinity-purified against its peptide coupled to Sepharose-4B, as described above.

Preparation of Aortic Tissue
Adult, female, retired breeder CD rats, ~8 to 10 months old and weighing 400 g (Charles River, Wilmington, Mass), were anesthetized with an intraperitoneal injection of sodium pentobarbital (33 mg in 750 μL). Heparin (600 U in 300 μL) in PBS was injected into the superior mesenteric artery to prevent clotting, and animals were perfused through the left ventricle with 10 mL of HBSS containing 1.8 mmol/L calcium (HBSSC) after transecting the vena cava. For Western blot analysis, the aorta was excised and placed on ice in HBSSC before isolation of the endothelium. For immunohistochemistry, the heparin wash was followed by perfusion fixation with 10 mL of 2% paraformaldehyde in HBSSC, and the aorta was dissected free of adhering connective tissue.

Immunohistochemistry
Fixed aortas were opened longitudinally, and 9-mm² samples taken at intervals along their length were prepared for microscopy. The samples were first incubated in 4% fish skin gelatin (Sigma Chemical Co), 1% normal goat serum, and 0.25% Triton X-100 in PBS (blocking buffer) for 30 minutes. This was followed by incubation for 1 hour at room temperature with either affinity-purified chicken antibody to Cx40 or Cx37 coupled to Sepharose-4B. Unbound material was then adsorbed with purified fusion proteins for either Cx40 or Cx37 coupled to Sepharose-4B. Specific antibodies were eluted from each column with 0.1 mol/L glycine, pH 2.5. Antisera against Cx43 were affinity-purified with the use of a synthetic peptide as previously described. The specificity of the anti-Cx40 antibody was confirmed by probing Western blots containing lysates of Xenopus oocytes injected with cRNAs for Cx37, Cx40, and Cx43. Specific labeling was observed only in lanes containing Cx40 (data not shown). Cx37 antibodies were characterized previously in a similar fashion. A mouse monoclonal antibody produced against the same peptide used by Beyer et al was purchased from Zymed. Because of the low titer of specific antibody in the ascites fluid provided, the monoclonal antibody was affinity-purified against its peptide coupled to Sepharose-4B, as described above.

Western Blot Analysis
The unfixed descending thoracic aorta was excised, opened longitudinally along the dorsal wall between the intercostal pairs from the...
and the abdominal aorta was exposed. The aorta was freed from the dorsal abdominal wall to facilitate placing the cuff or suture. In some experiments, a No. 10-0 polypropylene suture was placed ~3 mm above the aortic bifurcation, a region selected so that no branching vessels are immediately upstream or downstream from the coarct. A dissecting microscope with an ocular reticule was used so that consistent reductions in vessel diameter could be accomplished. In other experiments, a 2-mm segment of silicone tubing (inner diameter, 2 mm; outer diameter, 3 mm) was fitted around the aorta, and 2 sutures were placed to establish the desired degree of compression. Viscera were irrigated with 2 mL PBS before closure of the abdominal wall with No. 4-0 nylon. Superficial fascia was stapled, and the animals recovered under a warming lamp. At specified times after recovery, animals were killed, and aortas were harvested as described above. All animals were handled in accordance with institutional guidelines.

Results

Connexin Expression in the Endothelium of the Ventral Wall of the Thoracic and Abdominal Aortas

Relative connexin levels in endothelial cells of thoracic and abdominal aortas were analyzed by Western blot. A membrane preparation was isolated from the endothelium of the ventral wall of the thoracic aorta, extending from the aortic arch to the diaphragm. Microscopic examination of sections from extracted vessels indicated that the internal elastic membrane was not breached and that smooth muscle cells were intact. Thus, only endothelia were extracted. A similar preparation was made from abdominal aortic endothelium, between the left renal artery and the aortic bifurcation. This segment of the abdominal aorta was chosen because it lacks branching vessels, offering a continuous population of endothelial cells with a minimal contribution from those cells exposed to the stresses of flow at branch points. To facilitate comparison of different regions, each gel lane was loaded with proteins extracted from an area of the same size, presumed to contain similar numbers of cells.

Cx40 antibodies recognized a very closely spaced doublet of proteins on immunoblots of endothelial cell extracts from both thoracic and abdominal aortas (Figure 1). The doublet is routinely observed, although the bands do not completely resolve in the gel system used in Figure 1. Similar levels of...
Cx40 were found in thoracic and abdominal aortas. Duplicate blots probed for Cx37 displayed 2 bands (Figure 1). The more prominent band (arrowhead) exhibited a relative mobility corresponding to Cx37 expressed in oocytes (data not shown) and was present at similar levels in both samples. Affinity-purified antibodies to Cx43 recognized one major band on immunoblots of endothelial cell extracts from both the thoracic and the abdominal aortas. Densitometric analysis showed that there was ≈10-fold more Cx43 protein in the endothelium of the thoracic aorta than in the endothelium of the abdominal aorta.

Immunocytochemical analysis of connexin distribution was performed with whole mounts of aortic segments. After fixation, the vessels were slit longitudinally and flattened to provide an en face view of the intimal surface. This procedure allows the inspection of large continuous areas, which is not possible by cross sectioning. Staining of the ventral wall of the thoracic aorta, distal from branching collateral vessels, with affinity-purified anti-Cx40 antibodies revealed an intense punctate pattern that outlined individual endothelial cells. Cx40 staining was evident at the border of every cell with little or no cytoplasmic staining (Figure 2A). Immunostaining of the abdominal aorta with anti-Cx40 antibodies showed a similar pattern, with punctate staining around the periphery of each endothelial cell and minimal cytoplasmic labeling (Figure 2B). Similarly, Cx37 staining was apparent in each endothelial cell (Figure 2C and 2D). However, the number of puncta per cell and the spacing between the puncta appeared to be less uniform than in the case of Cx40.

Immunofluorescent staining of the endothelium for Cx43 was significantly different in thoracic versus abdominal aortas. The ventral wall of the thoracic aorta contained discrete groups of endothelial cells that stained positively for Cx43. Within these groups, a nonuniform distribution of Cx43 was observed around cell borders, and endothelium between the groups did not stain (Figure 2E). In contrast, the endothelial cells lining the ventral wall of the abdominal aorta displayed no detectable Cx43 staining (Figure 2F).

Localization of Cx43 at Aortic Branch Points and Flow Dividers

Immunohistochemical analysis revealed striking regional differences in Cx43 expression in the vasculature. Cx43 was prominently expressed in specific regions near the ostia of all aortic branches. For example, Cx43 was detected at the inferior edge of the opening of the celiac artery (Figure 3). Immunofluorescent staining of aortic endothelium began near the opening (dotted line) and was strong 5 to 7 rows of cells downstream, after which, it was sharply reduced. Cx43 was also strongly expressed by endothelial cells near the ostia of intercostal vessels branching from the dorsal wall of the thoracic aorta (Figure 4A). This staining was localized to aortic endothelium downstream from the vessel openings (Figure 4C). Directly upstream from an ostium, endothelial cells were mostly negative for Cx43 staining (Figure 4B). At the junction between the right renal artery and the aorta (Figure 5A), a similar pattern was observed. Aortic endothelium at the downstream edge of the ostium exhibited punctate labeling around the periphery of individual cells (Figure 5B). Flow dividers were also sites of increased Cx43 immunostaining. At the aortic bifurcation into the common iliac arteries (Figure 6A), endothelia covering the aortic carina were outlined by an intense punctate staining for Cx43 (Figure 6B). However, the endothelial lining of the common iliac artery, distal to the flow divider, was negative for Cx43 (Figure 6C, right common iliac).

Since intercellular channels formed from each vascular connexin exhibit distinctly different properties, it was important to determine whether the induction of Cx43 was accompanied by changes in the expression of the other connexins. Therefore, double-label immunolocalization was performed at the aortic bifurcation (Figure 7). Cx40 and Cx43 appeared to be extensively colocalized (Figure 7A and 7B), and Cx40 levels and distribution were not grossly different in cells containing Cx43 from cells without detectable Cx43 (data not shown). In contrast, Cx37 and Cx43 exhibited reciprocal patterns of expression; Cx37 was not detected in
regions where Cx43 was abundant (Figure 7C and 7D). Examination of other regions (data not shown) confirmed the general nature of these patterns.

**Induction of Cx43 by Aortic Coarctation**

The highly restricted pattern of Cx43 expression corresponds well to regions experiencing shear stress caused by nonlaminar flow conditions. Since shear stress is known to directly activate or repress a number of genes, it was of interest to more directly examine the relationship between shear stress and connexin expression. Therefore, flow disturbances in the aorta were created in vivo by surgical coarctation. In the first method, a surgical ligature was introduced in a segment of the abdominal aorta where Cx43 is not normally detected, reducing the vessel diameter by \( \approx 30\% \). After 8 days, animals were killed, and en face preparations were examined by double-label immunofluorescence microscopy (Figure 8). A strong accumulation of Cx43 was observed in the region corresponding the ligature (red, Figure 8A). Cx40 levels (green, Figure 8A) are essentially unchanged by this treatment, although its presence at the ligature is obscured by the strength of the Cx43 signal in Figure 8A. A significant amount of Cx40/Cx43 coexpression (yellow) is evident upstream but not downstream from the coarct. A separate preparation was double-labeled for Cx37 and Cx43 (Figure 8B). Unlike Cx40, Cx37 is strongly downregulated upstream from the coarct (green, Figure 8B), and as in the case of the aortic bifurcation (Figure 7), very little coexpression of Cx37 and Cx43 (yellow) was observed.

Since the effect on connexin expression might have resulted from arterial compression rather than hemodynamic forces, a second method of coarctation using a silicone tubing cuff was used. In these experiments, significant Cx43 expression was observed only at the leading and trailing edges of the cuff (data not shown), where pronounced nonlaminar flow is expected. Thus, increased expression of Cx43 and, possibly, decreased expression of Cx37 correlate with changes in hemodynamic forces in vivo.

**Discussion**

We have shown that at least 3 connexins are present in aortic endothelium but in different amounts and with dramatically different distributions. Cx40 was the most abundant connexin and was present in nearly all cells. Cx37 displayed a distribution generally similar to that of Cx40 but was less abundant. In contrast, Cx43 expression was very restricted. Cx43 was present in endothelial cells immediately downstream from the ostia of aortic branches and flow dividers. It was also detected in small groups of endothelia removed from ostia in the thoracic but not the abdominal aorta. In addition, we have shown that arterial coarctation results in a highly localized increase in levels of Cx43 with coincident reduction...
in levels of Cx37. Together, these data suggest that Cx43 is normally absent from quiescent large-vessel endothelia but is present when the vessel wall is subjected to shear stress.

The distribution of Cx43 that we observed conflicts with the recent report by Yeh et al., who readily detected it in aortic endothelium in vivo. One explanation for this discrepancy could be sampling error. In that study, immunostaining was performed on random cross sections without regard to their position of origin. Thus, it is possible that few abdominal segments sufficiently removed from branch points to eliminate Cx43 were examined. However, in previous experiments, we did not observe endothelial Cx43 in random aortic cross sections. Another explanation for the discrepancy could be strain-, age-, or diet-related differences in the tendency to develop Cx43-positive areas. In any case, since Yeh et al reported little or no Cx43 in coronary artery endothelia, our results are consistent to the extent that we both observe regional differences in Cx43 expression.

The distinctive localization of Cx43 at areas experiencing high levels of shear stress21-28 and the induction of Cx43 in response to experimentally imposed turbulence suggest shear stress regulation of Cx43. It has been shown that transcriptional activation of various genes by shear stress29 is conferred by a specific DNA element (shear stress response element [SSRE]).30 However, the putative rat Cx43 promoter region32 does not contain an exact match for the full (12-bp) or “core” (6-bp) SSRE sequence. Available 5’ non-coding sequences from the murine Cx43 gene also lack a match for the full SSRE, although several core sequences are present.33 More important, we have not directly demonstrated transcriptional activation, and our data are equally consistent with translational control of Cx43 synthesis or by alterations in the rates of mRNA or protein degradation. Therefore, it is possible that the assembly of junctions containing Cx43 is secondary to the activation of other genes controlled by SSREs or by mechanisms not involving SSRE-regulated gene expression. For example, Cx43 expression in the vascular wall could be regulated by growth factors such as basic fibroblast growth factor.34 It has been suggested that release of endogenous basic fibroblast growth factor by transient disruption of the plasma membrane causes the increase in Cx43 levels found in endothelial cultures after wounding.35 Since shear stress has been shown to transiently disrupt endothelial plasma membranes around ostia and at bifurcations,36,37 the localized increases in Cx43 observed in vivo could result from localized release of basic fibroblast growth factor.

How might the localized expression of the 3 connexins, particularly Cx43 in areas of shear stress, contribute to proper endothelial cell function? Regional differences in the expres-
sion of vascular connexins implies that although all endothelial communicate, they may not all do so equally. This hypothesis is consistent with in vitro studies of bovine microvascular endothelia, where regional differences in communication levels can be induced by mechanical trauma. Other connexin-intrinsic properties of communication, such as permeability and gating, might be affected. In addition, the expression of different connexins may vary either to limit the number of cells that are coupled or to compartmentalize groups of coupled cells. For example, it has been shown that cells expressing Cx43 readily form intercellular channels with each other but fail to establish functional channels with cells expressing Cx43 and Cx37, in conduit vessels, these incompatible connexins, Cx30 and Cx43, are primarily expressed in physically adjacent endothelial and smooth muscle cells, respectively, which may act to reduce or regulate the frequency of communication between these cells. In support of this notion, it is usually difficult to demonstrate coupling between endothelial and smooth muscle cells in vivo. In areas subject to injury or inflammation, endothelial cells may upregulate the expression of Cx43 to facilitate communication with other Cx43-expressing cell types, such as smooth muscle, monocytes, and macrophages, and lymphocytes. We speculate that the resultant new patterns of communication may influence the transition of quiescent endothelial cells to migratory or proliferative states for the repair of injury.

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References

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