Vascular Abnormalities in Mice Lacking the Endothelial Gap Junction Proteins connexin37 and connexin40

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Cells within the vascular wall are coupled by gap junctions, allowing for direct intercellular transfer of low molecular weight molecules. Although gap junctions are believed to be important for vascular development and function, their precise roles are not well understood. Mice lacking either connexin37 (Cx37) or connexin40 (Cx40), the predominant gap junction proteins present in vascular endothelium, are viable and exhibit phenotypes that are largely non-blood vessel related. Since Cx37 and Cx40 are coexpressed in endothelial cells and could overlap functionally, some roles of junctional communication may only be revealed by the elimination of both connexins. In this study, we interbreed Cx37 and Cx40 knockout mice to generate Cx37−/−/Cx40−/− animals and show that they display severe vascular abnormalities and die perinatally. Cx37−/−/Cx40−/− animals exhibit localized hemorrhages in skin, testis, gastrointestinal tissues, and lungs, with pronounced blood vessel dilatation and congestion occurring in some areas. Vascular anomalies were particularly striking in testis and intestine. In testis, abnormal vascular channels were present, with these channels coalescing into a cavernous, endothelium-lined blood pool resembling a hemangioma. These results provide evidence of a critical role for endothelial gap junction-mediated communication in the development and/or functional maintenance of segments of the mouse vasculature.

Key Words: connexin; gap junction; intercellular communication; endothelium; vascular abnormality; hemorrhage; hemangioma; vascular malformation.

INTRODUCTION

Vascular endothelial cells are coupled by gap junctions, structures which allow for the diffusional transfer of small molecules between adjacent cells. Gap junctions are clusters of intercellular channels, made up of connexin subunits, that are formed by the docking of hemichannels present in adjacent plasma membranes (Goodenough et al., 1996; Kumar and Gilula, 1996). In rodents, 19 connexins have been identified which have different functional properties and tissue-specific expression patterns (Willecke et al., 2002). Four connexins, Cx37, Cx40, Cx43, and Cx45, have been described in the vascular wall, which contains not only endothelial–endothelial gap junctions but also smooth muscle–smooth muscle and smooth muscle–endothelial gap junctions (Larson et al., 1990; Bruzzone et al., 1993; Reed et al., 1993; Little et al., 1995; Yeh et al., 1997; Gabriels and Paul, 1998; Traub et al., 1998; Ko et al., 1999; van Kempen and Jongsma, 1999; Krüger et al., 2000). Connexin expression is not identical in all blood vessels and connexin profiles in different parts of the vasculature have not been completely described. In addition, species-specific differences have been reported for connexin expression in some vessels. Most commonly, endothelial cells express Cx37 and Cx40, whereas smooth muscle cells express Cx43 or Cx45. Cx43 is also found in a subset of endothelial cells located near ostia and flow dividers in the rat vasculature (Gabriels and Paul, 1998) and may be expressed in endothelial cells of capillaries (Theis et al., 2001). Additional reports have described the presence of Cx37 or Cx40 in the vascular smooth muscle of specific blood vessels (Little et al., 1995; Traub et al., 1998; Li and Simard, 1999; Nakamura et al., 1999; Cai et al., 2001; Haefliger et al., 2001).

Several physiological roles have been proposed for vascular gap junction-mediated communication. Coupling is thought to be important for the conduction of vasomotor responses along arterioles and may also play a role in regulation of vascular tone in larger vessels (Segal and...
other vascular connexins (Cx37, Cx40, and Cx43). The role for gap junctions in vascular development was revealed in Cx45 knockout animals, which die between embryonic day 9.5 (E9.5) and E10.5. These animals exhibit impaired formation of yolk sac vasculature, abnormal placental vasculization, failure of smooth muscle formation around major arteries, as well as cardiac defects (Krüger et al., 2000; Kumai et al., 2000).

Knockout mouse models have also been produced for the other vascular connexins (Cx37, Cx40, and Cx43). The characterized phenotypes of Cx37−/−, Cx40−/−, and Cx43−/− mice are largely non-blood vessel related, in part because expression of these family members in wild-type mice is not restricted solely to the vasculature. Cx43 knockout mice, for example, die perinatally with abnormal development of the pulmonary outflow tract, due to aberrant migration of cardiac neural crest cells, which normally express Cx43 and contribute to outflow tract development (Reaume et al., 1995; Ya et al., 1998; Lo et al., 1999). Recently, viable endothelial-specific knockouts of Cx43 were generated independently by two groups, each using the Tie 2 promoter to direct endothelium-specific gene ablation (Liao et al., 2001; Theis et al., 2001). Liao et al. (2001) reported that these mice exhibit hypotension and bradycardia, suggesting that there are endothelial-specific roles for Cx43. Theis et al. (2001), however, found that the absence of Cx43 in endothelium did not alter blood pressure or heart rate. The discrepancy between these results is not understood at present. In contrast to germline Cx43 knockout animals, mice lacking Cx37 are viable, but are female infertile because of abnormal development of both oocytes and ovarian follicles (Simon et al., 1997). Consistent with this phenotype, Cx37 is found in gap junctions between oocytes and granulosa cells. Finally, ablation of Cx40 results in mice with prolonged atrioventricular conduction, right bundle branch block, and a predisposition for arrhythmias, in concordance with expression of Cx40 in atrium and cardiac conduction system (Kirchhoff et al., 1998; Simon et al., 1998). Cx40 knockout animals have also been reported to exhibit diminished conduction of arteriolar dilatation in response to acetylcholine and bradykinin and are hypertensive, but do not have other obvious blood vessel abnormalities (de Wit et al., 2000). Thus, despite prominent expression of Cx37 and Cx40 in endothelial cells, ablation of either of these connexins separately does not result in a severe vascular phenotype, and in particular, blood vessel developmental defects are not observed. Since endothelial cells coexpress Cx37 and Cx40, it is possible that these connexins overlap functionally in the endothelium and that some vascular phenotypes will only be observed when both connexins are eliminated.

In this report, we describe the generation and characterization of mice lacking both of the predominant vascular endothelial connexins, Cx37 and Cx40, and provide evidence that gap junctional communication, and specifically endothelial coupling, is important for the normal development and/or functional maintenance of portions of the mouse vasculature. While mice lacking either of these connexins separately are viable, Cx37−/−Cx40−/− mice do not survive past the first postnatal day (P1) and exhibit severe vascular abnormalities in skin, testis, intestine, stomach, and lung.

**MATERIALS AND METHODS**

**Mouse Breeding Strategy**

Cx37−/−Cx40−/− mice were obtained by interbreeding Cx37−/− and Cx40−/− mice (Simon et al., 1997, 1998). Male Cx37−/− mice on a mixed 129/Sv-C57BL/6 background were interbred with female Cx40−/− mice (129/Sv-C57BL/6 background). Cx37−/−Cx40−/− offspring were backcrossed with Cx40−/− mice. Cx37−/−Cx40−/− offspring were interbred to obtain Cx40−/−, Cx37−/−Cx40−/−, and Cx37−/−Cx40−/− animals at various developmental stages. Cx37−/− animals were obtained by interbreeding Cx37−/− males with Cx37−/− females. Although data presented in this report are derived from the mixed strain mice, we have obtained similar results with C57BL/6 strain mice.

**PCR Genotyping**

Wild-type and knockout connexin alleles were detected by PCR. Cx37 wild-type and knockout alleles were amplified with the

Vascular Abnormalities in Cx37−/−Cx40−/− Mice

Duling, 1989; Christ et al., 1996; Chaytor et al., 1998; Emerson and Segal, 2000). In addition, cell culture studies suggest that junctional coupling may be important in the control of vascular cell proliferation and migration during development or wound healing (Larson and Haudenschild, 1988; Pepper et al., 1989; Kurjiaka et al., 1998). Recently, the expression of these family members in wild-type mice is not restricted solely to the vasculature. Cx43 knockout mice, for example, die perinatally with abnormal development of the pulmonary outflow tract, due to aberrant migration of cardiac neural crest cells, which normally express Cx43 and contribute to outflow tract development (Reaume et al., 1995; Ya et al., 1998; Lo et al., 1999). Recently, viable endothelial-specific knockouts of Cx43 were generated independently by two groups, each using the Tie 2 promoter to direct endothelium-specific gene ablation (Liao et al., 2001; Theis et al., 2001). Liao et al. (2001) reported that these mice exhibit hypotension and bradycardia, suggesting that there are endothelial-specific roles for Cx43. Theis et al. (2001), however, found that the absence of Cx43 in endothelium did not alter blood pressure or heart rate. The discrepancy between these results is not understood at present. In contrast to germline Cx43 knockout animals, mice lacking Cx37 are viable, but are female infertile because of abnormal development of both oocytes and ovarian follicles (Simon et al., 1997). Consistent with this phenotype, Cx37 is found in gap junctions between oocytes and granulosa cells. Finally, ablation of Cx40 results in mice with prolonged atrioventricular conduction, right bundle branch block, and a predisposition for arrhythmias, in concordance with expression of Cx40 in atrium and cardiac conduction system (Kirchhoff et al., 1998; Simon et al., 1998). Cx40 knockout animals have also been reported to exhibit diminished conduction of arteriolar dilatation in response to acetylcholine and bradykinin and are hypertensive, but do not have other obvious blood vessel abnormalities (de Wit et al., 2000). Thus, despite prominent expression of Cx37 and Cx40 in endothelial cells, ablation of either of these connexins separately does not result in a severe vascular phenotype, and in particular, blood vessel developmental defects are not observed. Since endothelial cells coexpress Cx37 and Cx40, it is possible that these connexins overlap functionally in the endothelium and that some vascular phenotypes will only be observed when both connexins are eliminated.

In this report, we describe the generation and character-
following primers: primer 1, 5’-TGCTAGACCAGCTCCAG-GAAC-3’; primer 2, 5’-AGAGGCTATTCGGCTATGACTG-3’; and primer 3, 5’-GTCCCTTCGCTCTTTATCTC-3’. Primers 1 and 2 amplify a 1.3-kb fragment from the Cx37 knockout allele. Primers 1 and 3 amplify a 0.75-kb fragment from the Cx37 wild-type allele. Thirty cycles were performed by using Titanium Taq enzyme (Clontech) with the following parameters: 94°C denaturation (30 s), 63°C annealing (30 s), 72°C extension (90 s). Cx40 wild-type and knockout alleles were amplified with the following primers: primer 4, 5’-TGGAGCCACAGTTGCAATGGT-3’; primer 5, 5’-GCACGAGACTAGTGAGACGTG-3’; primer 6, 5’-TCTCTGACTCCGAAAGGCAAG-3’. Primers 4 and 5 amplify a 470-bp fragment from the Cx40 knockout allele. Primers 4 and 6 amplify a 270-bp fragment from the Cx40 wild-type allele. Cycling conditions were the same as the Cx37 protocol with the exception that the annealing temperature was 64°C and the extension time was 30 s.

Immunohistochemistry

Mouse tissues were frozen unfixed in Tissue Tek OCT and sectioned at 10-μm thickness. Before immunostaining, sections were fixed in acetone at −20°C for 5 min and then blocked in a solution containing phosphate-buffered saline, 4% fish skin gela-

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Total no. of animals</th>
<th>Cx40+/−</th>
<th>Cx37+/−Cx40+/−</th>
<th>Cx37+/−Cx40−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5</td>
<td>33</td>
<td>11 (33%)</td>
<td>15 (46%)</td>
<td>7 (21%)</td>
</tr>
<tr>
<td>E18.5</td>
<td>285</td>
<td>79 (28%)</td>
<td>146 (51%)</td>
<td>60 (21%)</td>
</tr>
<tr>
<td>P0-P1</td>
<td>174</td>
<td>45 (26%)</td>
<td>96 (55%)</td>
<td>33 (19%)</td>
</tr>
<tr>
<td>P2</td>
<td>49</td>
<td>15 (31%)</td>
<td>34 (69%)</td>
<td>0</td>
</tr>
<tr>
<td>3-wk postnatal</td>
<td>421</td>
<td>154 (37%)</td>
<td>267 (63%)</td>
<td>0</td>
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</table>

FIG. 2. Subcutaneous hematomas and vessel dilation in newborn and E18.5 Cx37+/−Cx40−/− mice. The external appearance of a newborn Cx37+/−Cx40−/− pup (A) and Cx40−/− littermate (B) revealed subcutaneous hematomas (arrows) in the double knockout animal. Hematomas also were observed in Cx37+/−Cx40−/− embryos at E18.5 (C) but not in Cx40−/− littermates (D). Cx37+/−Cx40−/− animals also exhibited dilated skin vessels (E) in some affected areas. Dilated vessels were not present in Cx40−/− littermates (F). Hematoxylin–eosin-stained paraffin section from the neck (G) of an E18.5 Cx37+/−Cx40−/− embryo showing subcutaneous accumulation of extravascular blood (arrow). Scale bar, 500 μm.

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tin, 1% normal goat serum, 0.25% Triton X-100, for 20 min. Anti-connexin antibodies (0.5 mg/ml) were diluted 1/150–1/300 in blocking solution and incubated on sections for 2 h at room temperature. Anti-Cx37, anti-Cx40, and anti-Cx43 sera were kindly provided by David Paul (Harvard Medical School) and have been described previously (Beyer et al., 1987; Gabriels and Paul, 1998). To identify blood vessels, sections were coabeled with an anti-PECAM-1 antibody (Pharmingen; clone MEC 13.3), diluted at 1/200. For negative controls, the primary antibody (or both primary and secondary antibody) was omitted on nearby sections. Sections were washed and then incubated for 1 h with CY3- or CY5-conjugated AffiniPure Donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch) to detect connexin labeling and FITC-conjugated AffiniPure Donkey anti-rat IgG (H + L) (Jackson ImmunoResearch) to detect PECAM-1 labeling. After washing, sections were viewed with an Olympus BX51 fluorescence microscope. Images were captured with a SensSys 1401 CCD camera (Photometrics) and V++ software. Immunostaining with rabbit antibodies against claudin-5 (Zymed cat no. 34-1600), occludin (Zymed cat no. 71-1500), and ZO-1 (Zymed cat no. 61-7300) was performed in

FIG. 3. Testicular vascular defects in E18.5 Cx37−/−Cx40−/− embryos. Gross appearance of testis from an E18.5 Cx37−/−Cx40−/− embryo (A) and a wild-type control testis (B), showing a large blood pool in the double knockout testis. (C) Hematoxylin–eosin-stained paraffin section of double knockout testis, revealed numerous abnormal vascular channels (arrows) in between testicular cord tissue. (D) Higher magnification image of an abnormal vascular channel, lined with endothelium (arrow). (E) Vascular channels coalesced into a large blood pool (asterisk) in the central region of the Cx37−/−Cx40−/− testis. (F) Higher magnification view of the section in (E), showing blood pool (asterisk) and localized area of hemorrhage. Diffuse vacuolar degeneration and necrosis of testicular tissue was evident immediately to the right. (G) Section of E18.5 wild-type control testis, showing absence of vascular defects. (H) Electron microscopy of Cx37−/−Cx40−/− testis, showing a congested vascular channel lined with an endothelium. (I) Section of E14.5 Cx37−/−Cx40−/− testis with abnormal vascular channels (arrows). (J) Section of E14.5 testis from a Cx40−/− littermate control with no vascular abnormalities. Scale bars, 50 μm for (C, E, F, G); 20 μm for (H); and 10 μm for (D, I, J).
a similar fashion, with dilutions of 1/200, 1/200, and 1/400, respectively.

Histological Analysis

Specimens were fixed in 4% paraformaldehyde before processing for paraffin sectioning. Samples were embedded in paraplast and serially sectioned, transversely, at 10-μm thickness before staining with hematoxylin–eosin. Intestines were collected in Karnovsky’s fixative, embedded in methacrylate, sectioned at 0.5-μm thickness, and stained with either a polychrome mixture, Toluidine Blue, or Methylene Blue. Silver nitrate staining of vascular endothelial cells was performed by a modification of the method described by McDonald (1994). Vascular permeability was tested by slowly

### TABLE 2

Frequency of Vascular Defects in Cx37−/− Cx40−/− E18.5 Embryos and Newborn Pups

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>E18.5</th>
<th>P0-P1</th>
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<tbody>
<tr>
<td></td>
<td>Cx40−/−</td>
<td>Cx37−/− Cx40−/−</td>
</tr>
<tr>
<td>Subcutaneous hematoma</td>
<td>2/81</td>
<td>7/140</td>
</tr>
<tr>
<td>Testicular vascular defect</td>
<td>0/24</td>
<td>0/23</td>
</tr>
<tr>
<td>Intestinal or gastric vascular defect</td>
<td>0/35</td>
<td>1/40</td>
</tr>
<tr>
<td>Lung hemorrhage</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mean embryo weight</td>
<td>1.00 ± 0.03 g (n = 55)</td>
<td>0.94 ± 0.02 g (n = 116)</td>
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Note. For the first four rows, the number of animals with the indicated phenotype per total number of animals analyzed is presented. nd, not determined.

FIG. 4. Expression of endothelial markers in testis of E18.5 Cx37−/− Cx40−/− and wild-type embryos. Immunostaining for proteins typically expressed by endothelial cells revealed abnormal distribution of endothelial cells in Cx37−/− Cx40−/− testis. Cx37−/− Cx40−/− testis sections (A, B, E, F, I, J) and wild-type sections (C, D, G, H, K, L) were immunostained for PECAM (A–D), claudin-5 (E–H), and caveolin-1 (I–L). (B, D, F, H, J, L) Fourfold higher magnification images than (A, C, E, G, I, K). In Cx37−/− Cx40−/− sections, the antibodies labeled cells lining abnormal vascular channels and blood pools, whereas in wild-type sections, labeling occurred in smaller vessels dispersed throughout the tissue and in testicular arteries. Examples of abnormal blood-filled regions in Cx37−/− Cx40−/− testis are marked by asterisks. In addition to altered spatial distribution, the intensity of PECAM staining in Cx37−/− Cx40−/− testis was elevated relative to wild-type testis. Scale bars, 50 μm.
injecting 25 μl of India ink into the left ventricle of E18.5 embryos. After a few minutes, the ink-injected embryos were fixed and organs embedded for paraffin sectioning and hematoxylin–eosin staining.

RESULTS

Perinatal Lethality in Cx37+/− Cx40+/− Mice

We produced mice lacking both Cx37 and Cx40 by interbreeding Cx37+/− and Cx40+/− mouse lines (Simon et al., 1997, 1998). Cx37+/− Cx40+/− offspring from the initial cross were backcrossed with Cx40+/− animals and the litters screened for Cx37+/− Cx40+/− mice, which were born at expected frequencies and were viable and fertile. Cx37+/− Cx40+/− animals were then interbred and the resulting litters were genotyped using 3-primer PCR assays at various stages of prenatal and postnatal development (Figs. 1A and 1B). The expected Mendelian frequency of Cx37+/− Cx40+/− animals from this cross is 25%. At E14.5, E18.5, and P0–P1, Cx37+/− Cx40+/− offspring were identified at frequencies of 21, 21, and 19%, respectively (Table 1), suggesting a slight reduction in viability of Cx37+/− Cx40+/− embryos at E14.5. A more striking reduction in viability occurred around birth and during P1, such that by P2 all of the Cx37+/− Cx40+/− animals had died (Table 1). Many Cx37+/− Cx40+/− animals were alive at birth and survived for several hours, but expired at variable times during P1. No Cx37+/− Cx40+/− animals were found among 3-week-old weanlings (421 animals analyzed).

Vascular Phenotypes of Cx37+/− Cx40+/− Mice

Newborn Cx37+/− Cx40+/− mice appeared fully developed in general, but displayed severe vascular abnormalities in a subset of tissues (skin, testis, intestine, stomach, and lung). In contrast, other organs, such as brain, kidney, and liver, did not have obvious vascular defects, and the major systemic vessels appeared normal. Externally, Cx37+/− Cx40+/− mice exhibited subcutaneous hematomas that were typically located along the dorsal surface of the animal, especially on the head, but also on the neck, shoulder, and posterior regions (Fig. 2A, arrows). In addition, dilation of blood vessels in the skin near affected areas was usually evident (Fig. 2E). Greater than 90% (30/33) of newborn Cx37+/− Cx40+/− animals exhibited obvious subcutaneous hematomas, whereas none were observed with newborn wild-type, Cx40+/−, or Cx37+/− mice (Table 2). At the time of collection, the severity and pattern of hematomas varied from animal to animal, presumably reflecting differences in the timing of phenotypic progression. Subcutaneous hematomas were also observed at a high frequency in Cx37+/− Cx40+/− embryos at E18.5 (73/76 embryos), indicating that the phenotype was not simply a result of birth trauma (Fig. 2C and Table 2). At E18.5, mean embryo weight of Cx37+/− Cx40+/− animals was slightly less than control littersmates (Table 2). Histological sections through head and neck (Fig. 2G) of E18.5 Cx37+/− Cx40+/− embryos showed subcutaneous extravascular blood (arrow) in areas where hematomas were noted before sectioning. At E14.5, Cx37+/− Cx40+/− embryos appeared externally similar to Cx40+/− and Cx37+/− Cx40−/− littermates, indicating that subcutaneous hematomas arise sometime after E14.5. Male Cx37+/− Cx40+/− mice exhibited a striking testicular vascular anomaly which presented as a variable sized blood-filled region comprising up to more than half the organ volume (Fig. 3A). All of the double knockout males examined at E18.5 had this testicular phenotype (16/16), whereas none were observed in male Cx40+/− and Cx37+/− Cx40+/− littermates (Table 2). In the outer part of the testis, serial histological sections revealed the presence of congested, thin-walled vascular channels in between testicular cord tissue (Figs. 3C and 3D). In deeper sections, these abnormal structures coalesced into a cavernous blood pool (Figs. 3E and 3F asterisk). Blood pools were extensively lined with vascular endothelial cells, which were identified by strong expression of the endothelial marker, PECAM-1 (Figs. 4A and 4B). The intensity of PECAM-1 immunostaining in the Cx37+/− Cx40+/− endothelial cells, which were often closely associated with the remaining testicular cord tissue, appeared elevated compared with blood vessels in control testis (Figs. 4A–4D). The PECAM-1-positive cells also expressed claudin-5, ZO-1, VE-cadherin, and caveolin-1, proteins that are typically expressed by vascular endothelial cells (claudin-5 and caveolin-1 immunostaining is shown in Figs. 4E–4L). Immunostaining of Cx37+/− Cx40+/− testis for these endothelial markers resulted in labeling of cells lining the abnormal vascular channels or blood pools, whereas in wild-type testis, labeling occurred in smaller vessels that were dispersed throughout the tissue. Electron microscopy of Cx37+/− Cx40+/− testis confirmed the presence of an endothelium lining the vascular anomalies (Fig. 3H). The pattern of endothelial markers in Cx37+/− Cx40+/− testis suggested that the vascular defects observed in testis were the result of dysmorphogenesis, at least initially, rather than simple hemorrhage. Abnormal vascular channels were observed in double knockout testis as early as E14.5, though the defects at this stage were not as large or numerous as in E18.5 testis (Figs. 3I and 3J). These results are consistent with a developmental defect that begins around E14.5 and progresses into later fetal stages. In some sections of E18.5 testis, we observed areas of hemorrhage where damage presumably occurred to the abnormal vascular structures (Fig. 3F). Finally, in addition to the vascular defects described above, mild to severe diffuse spermatagonia vacuolar degeneration and necrosis was observed in some areas of the remaining testicular tissue (Fig. 3F), which could be due to inefficient circulation in the tissue. None of these defects were observed in testicular sections from littermate controls or wild-type animals (Figs. 3B, 3G, and 3J). Pronounced vascular anomalies were also observed in the gastrointestinal tracts of Cx37+/− Cx40+/− animals analyzed at E18.5. Upon dissection, 76% of Cx37+/− Cx40+/− embryos (39/51 examined) had obvious vascular abnormalities in small intestine and/or stomach, whereas none of the Cx40+/− littermates and only one of the Cx37+/− Cx40+/−
FIG. 5. Intestinal vascular defects in E18.5 Cx37−/−Cx40−/− embryos. (A) Gross appearance of duodenum from an E18.5 Cx37−/−Cx40−/− embryo, showing underlying redness in the intestinal wall and pronounced dilatation of surface blood vessels. (B) Intestine from a Cx40−/− littermate control. (C) Methylene Blue-stained plastic section of double knockout duodenum revealing abnormally large, distended vessels in the submucosa (arrows), with prominent congestion of blood, as well as hemorrhage in some areas. Erythrocytes appear greenish blue in these sections. (D) Plastic section of a Cx40−/− littermate control. A submucosal vessel is indicated by an arrow. (E) A higher magnification image of an abnormal vessel from a section of Cx37−/−Cx40−/− intestine, showing endothelium (arrow) with no obvious mural cell association. (F) Congestion and hemorrhage as well as necrosis were observed in the lamina propria of intestinal villi. (G) PECAM-1 immunostaining of a frozen cross-section of Cx37−/−Cx40−/− intestine, showing more extensive staining compared with a
affected regions tended to be more severe in the proximal portion of the small intestine and were characterized by underlying redness in the intestinal or gastric wall and pronounced enlargement of surface blood vessels (Fig. 5A). Histological sections of duodenum revealed abnormally large, distended vessels in the submucosa (Fig. 5C, arrows), with prominent congestion of blood in these vessels, as well as hemorrhage in other areas. PECAM-1 immunostaining of Cx37<sup>-/-</sup>/Cx40<sup>-/-</sup> intestinal frozen sections showed a more extensive vessel pattern compared with littermates or wild-type controls (Figs. 5G and 5H). Distended intestinal vessels had thin walls consisting of a single layer of endothelial cells, with no obvious mural cell association, and reached diameters of greater than 150 μm (Fig. 5E, arrow). Vascular defects were also observed in the lamina propria of intestinal villi (Fig. 5F). In some areas, mild multifocal hemorrhage and necrosis were observed in the lamina propria, whereas in other areas, severe diffuse hemorrhage was evident. Vascular defects were not seen in sections of intestine from Cx40<sup>-/-</sup> or Cx37<sup>-/-</sup>/Cx40<sup>-/-</sup> littermate controls (Figs. 5D and 5H).

In addition to the above vascular defects, a significant proportion (45%) of Cx37<sup>-/-</sup>/Cx40<sup>-/-</sup> newborns had extravascular blood in their lungs, partially filling the airspaces (Figs. 6A and 6C, and Table 2). By comparison, this phenotype was observed in only 10% of wild-type, 11% of Cx37<sup>-/-</sup> animals, and 23% of Cx40<sup>-/-</sup> animals, indicating that lung hemorrhage was predominantly associated with the loss of both connexins (Table 2).

**Cx37, Cx40, Cx43 Immunostaining in Wild-Type E18.5 Skin, Testis, Intestine, Lung**

Connexin expression in blood vessels has been examined in a number of species, but expression in the mouse vasculature has not been completely described for many tissues. Since vascular abnormalities were observed in skin,
FIG. 7. Cx37, Cx40, and Cx43 immunostaining in skin, testis, intestine, and lung of E18.5 wild-type embryos. Frozen sections of E18.5 wild-type skin (A–F), testis (G–L), intestine (M–R), and lung (S–X) were double labeled with anti-Cx37, anti-Cx40, or anti-Cx43 antibody and anti-PECAM-1 antibody. (A, G, M, S) Anti-Cx37 immunolabeling; (C, I, O, U) Anti-Cx40 immunolabeling; (E, K, Q, W) Anti-Cx43 immunolabeling; (B, D, F, H, J, L, N, P, R, T, V, X) Anti-PECAM-1 immunolabeling. In skin, Cx37 and Cx40 are expressed in the endothelium of a subset of subcutaneous PECAM-1-positive vessels. Cx43 is not expressed in endothelium of skin vessels but is expressed in keratinocyte cell layers and hair follicles of the epidermis. In testis, Cx37 and Cx40 were strongly expressed in the testicular artery and more weakly expressed in smaller vessels. Some PECAM-1-positive vessels did not express Cx37 or Cx40. Cx43 was absent from the endothelium of testicular vessels but was strongly expressed by Sertoli cells and more weakly by cells in the tunica albuginea. In intestine, Cx37 and Cx40 were strongly expressed in a subset of submucosal vessels. Weak Cx43 immunostaining was more widespread in the intestinal sections, but was absent from the endothelium of PECAM-1-positive submucosal vessels. In lung, Cx37 and Cx40 were strongly expressed in the endothelium of pulmonary artery and its branches. Cx40, but not Cx37, was observed in the lung parenchyma. Cx43 immunostaining was not observed in pulmonary artery endothelium but was found in vessels characteristic of lymphatics. Scale bar, 50 μm.
Cx37 and Cx40 immunostaining was very strong in the endothelium of the testicular artery at E18.5, both at the periphery of the testis and in more central locations, and more weakly present in a subset of smaller testicular vessels (Figs. 7G–7L). As in skin, some PECAM-1-positive vessels did not show detectable Cx37 or Cx40 staining. In adult testis, Cx37 and Cx40 labeling was very weak or absent in the testicular artery, indicating a developmental change in connexin expression in this artery (not shown). Endothelial Cx43 expression was absent in both E18.5 and adult testicular artery. Because the anti-Cx37 and anti-Cx40 antibodies used in this study were both raised in rabbit, we were not able to double label for these connexins. Staining of immediately adjacent sections, however, indicated that the same vessels expressed both Cx37 and Cx40 in endothelium. In addition, when serial sections of individual vessels were stained for either Cx37 or Cx40, expression was found to be continuous along the length of the vessel. Thus, Cx37 and Cx40 are very likely coexpressed within endothelial cells in the tissues we examined, consistent with prior studies of connexin expression in the vasculature (Yeh et al., 1998; Ko et al., 1999).

Cx37 and Cx40 antibodies also labeled endothelium of a subset of PECAM-1-positive blood vessels in the E18.5 intestine (Figs. 7M–7R). Staining was strong in submucosal vessels present near the periphery of the intestine cross-sections and weaker in smaller vessels located in the lamina propria. Weak Cx43 immunostaining was more widespread in the intestinal sections, with labeling occurring on cells in the submucosa and lamina propria. Although the labeling pattern for Cx43 was complicated, PECA-1-positive arterioles in the submucosa were clearly negative for Cx43 in the endothelial layer.

In sections of E18.5 lung, Cx37 and Cx40 immunostaining was strong in the endothelium of the pulmonary arteries and its branches (Figs. 7S–7X). Punctate Cx40 labeling, but not Cx37, was also observed in the lung parenchyma. Cx43 staining was not observed in the endothelium of pulmonary arteries, but was detected in the endothelium of nearby vessels that were characteristic of lymphatics.

Finally, we immunostained Cx37+/−Cx40+/− sections for Cx43 and found no compensatory changes in Cx43 expression in vascular endothelium or other cell types (Fig. 8). As expected, Cx40 and Cx37 immunostaining was absent in all E18.5 Cx37+/−Cx40+/− tissues examined, including blood vessels (not shown).

**Endothelial Cell-Cell Contacts, Tight Junction Protein Expression and Vascular Permeability in Cx37+/−Cx40+/− Vessels**

To test whether endothelial cells established normal cell-cell contacts and morphology, silver nitrate staining and electron microscopy were performed on tissues from E18.5 embryos. Silver nitrate stains material in the intercellular space, outlining endothelial cells and marking gaps between cells when observed en face (McDonald, 1994). Endothelial cell-cell contacts and cell shape in Cx37+/−Cx40+/− intestinal...
blood vessels appeared similar to control specimens after silver nitrate staining (Figs. 5I and 5J). Gaps between endothelial cells were not observed in either Cx37/Cx40 knockout or control vessels. We also examined thin sections of E18.5 Cx37/Cx40 knockout intestine by electron microscopy and found normal contacts between endothelial cells and structures that were characteristic of adherens junctions and tight junctions (Figs. 5K and 5L). Our electron microscopy analysis of Cx37/Cx40 knockout intestinal vessels was not extensive, however, and we cannot rule out the possibility that some endothelial gap junctions, containing connexins other than Cx37, Cx40, and Cx43, are still present in these vessels.

Cryosections of affected tissues were stained for tight junction proteins to test for the possibility that the loss of vascular gap junctions caused changes in other types of intercellular junctions in endothelial cells. Sections of E18.5 intestine, testis, and lung were immunostained for the tight junction proteins claudin-5, occludin, and ZO-1 (data for testis are shown in Fig. 9). In wild-type tissues, vascular endothelial cells were strongly stained for claudin-5 and ZO-1. Occludin staining in endothelium was generally weak except for testicular artery, where the staining was very intense. In all cases, expression of these markers was qualitatively normal in Cx37/Cx40 knockout blood vessels, suggesting the continued presence of tight junctions. We noted elevated and more apically located ZO-1 staining in the intestinal epithelium of Cx37/Cx40 knockout animals when compared with wild-type tissue, presumably reflecting a stress-induced response to the vascular defects in this tissue.

We tested the possibility that blood vessels in Cx37/Cx40 knockout animals were abnormally leaky, even before hemorrhage, by injecting India ink as a particulate permeability tracer. Ink particles, with a diameter of ~30–60 nm, are too large to pass through normal endothelium, except following treatment with agents such as VEGF, histamine, or serotonin, which are known to increase permeability (Feng et al., 1997). Ink was introduced into the vasculature of E18.5 embryos by injection into the left ventricle and allowed to circulate before fixing and sectioning tissues. We examined the ink distribution in blood vessels that were away from hemorrhaged areas. In both duodenum and testis, ink particles were restricted to the lumen of vessels, similar to the pattern observed with littermate controls (Fig. 10). These results indicate that double knockout vessels were not abnormally permeable to this particulate tracer.

**DISCUSSION**

We generated mice lacking both vascular endothelial gap junction proteins, Cx37 and Cx40, and find that these animals die perinatally with vascular abnormalities in a subset of tissues, namely, skin, testis, intestine, stomach, and lung. Previous studies have suggested that gap junction-mediated communication is important for vasomotor re-
sponses, vascular proliferation and migration, and vascular development. Our results provide strong evidence of an important role for junctional communication, and specifically vascular endothelial coupling, in the development and/or functional maintenance of portions of the mouse vasculature. At birth, Cx37−/− Cx40−/− animals exhibit a highly penetrant phenotype of vascular dysmorphogenesis, congestion, and hemorrhage in affected tissues. A likely cause of death is inadequate pulmonary function caused by lung hemorrhage, although bleeding in other tissues probably also contributes.

Some phenotypic similarities between Cx45−/− and Cx37−/− Cx40−/− animals are evident, but there are also major differences in their vascular phenotypes (Krüger et al., 2000). Both genotypes show abnormally enlarged blood vessels (e.g., vessels of skin and intestine in E18.5–P1 Cx37−/− Cx40−/− embryos and placental capillaries in E9.5 Cx45−/− embryos). Unlike Cx37−/− Cx40−/− animals, Cx45−/− embryos also display narrowing of some vessels, such as portions of the dorsal aortae. In addition, both genotypes exhibit endothelium-lined cavernous vascular spaces (e.g., testis of E18.5 Cx37−/− Cx40−/− embryos and yolk sac of E9.5 Cx45−/− embryos). Due to critical defects in all three embryonic blood vessel systems, however, Cx45−/− animals die much earlier than Cx37−/− Cx40−/− animals (E9.5–E10.5 for Cx45−/− embryos vs P0–P1 for Cx37−/− Cx40−/− animals). Cx45−/− embryos undergo normal vasculogenesis, but subsequent steps of vascular remodeling are deficient and the embryos exhibit impaired formation of yolk sac vasculature, defective placental vascularization, and failure of smooth muscle formation around major arteries. In contrast, Cx37−/− Cx40−/− animals do not have obvious defects in yolk sac, placenta, or

FIG. 10. Permeability of Cx37−/− Cx40−/− blood vessels. India ink was injected into the vasculature of a Cx37−/− Cx40−/− E18.5 embryo and a littermate control embryo (Cx37−/− Cx40−/−) via the left ventricle and the distribution of ink particles observed after paraffin sectioning testis (A, B) and intestine (C–F). Nonhemorrhaged areas were examined for extravascular ink as evidence of increased permeability of microvessels in Cx37−/− Cx40−/− tissues. Ink particles were restricted to the lumen of microvessels (arrows), indicating that double knockout vessels were not abnormally permeable to this particulate tracer. In (C) and (D), capillaries in the lamina propria of intestinal villi are shown. In (E) and (F), ink-filled capillaries in the intestinal submucosa are shown. Sections were counterstained with hematoxylin–eosin. Scale bar, 10 μm.

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The distinct phenotypes of Cx45+/− and Cx37−/−Cx40−/− mice may reflect differences in cell type-specific expression of the connexins. Krüger et al. (2000) have described the expression of a lacZ reporter gene under the control of the endogenous Cx45 promoter, providing useful information about expression of the Cx45 gene. Strong β-gal staining was detected in vascular smooth muscle cells of postnatal day 2 (P2) animals and adult mice but only occasional weak staining in endothelium. At an earlier stage, however, β-gal staining was observed almost everywhere in E9.5 embryos, presumably including vascular endothelium. Thus, in blood vessels, Cx45 is initially expressed in both smooth muscle cells and endothelium but is eventually restricted mainly to smooth muscle cells. It is not presently known at what stage Cx45 expression is first turned off in vascular endothelium. Therefore, firm conclusions about the role of endothelial vs smooth muscle gap junctional communication in explaining differences in the Cx45+/− and Cx37−/−Cx40−/− phenotypes cannot be made. Possibly, Cx45 is no longer expressed significantly by endothelial cells during the stages examined in this study (E14.5–P1), potentially explaining why Cx45 is unable to compensate for a deficiency in endothelial Cx37 and Cx40.

Why are vascular abnormalities observed in only certain tissues in Cx37−/−Cx40−/− animals? Immunostaining with anti-connexin antibodies showed that Cx37 and Cx40 are expressed by vascular endothelial cells in wild-type blood vessels of affected tissues, as would be expected. These connexins are also expressed in blood vessels in many other tissues, however, where vascular abnormalities were not observed in Cx37−/−Cx40−/− animals. One possible explanation for the restricted phenotype is that defects develop in blood vessels where Cx37 and Cx40 are the only endothelial connexins and where there are no compensatory changes in connexin expression. Consistent with this idea, we did not observe Cx43 immunostaining in the endothelium of wild-type blood vessels of affected tissues. Furthermore, compensatory expression of Cx43 was not observed in Cx37−/−Cx40−/− vessels. It remains to be determined whether vessels in nonaffected tissues express other connexins or undergo compensatory expression. Alternatively, the specific vascular defects in Cx37+/−Cx40−/− animals may highlight tissues where gap junction-mediated communication is particularly critical for blood vessel development or function during fetal development, or where local environmental stresses play a significant factor. In this regard, it is interesting that Cx37 and Cx40 are strongly expressed in the testicular artery in the E18.5 embryo but are absent in the adult testicular artery, consistent with an important role for Cx37 and Cx40 during testicular vascular development. Previous studies have noted that Cx37 levels are generally higher in embryonic organs than in corresponding adult organs (Traub et al., 1998). In some tissues, however, vascular communication may be more important during postnatal or adult stages than embryonic stage. Thus, vascular defects might have become evident in other tissues, if the double knockout animals survived past the first postnatal day. Finally, tissues that were unaffected by the ablation of Cx37 and Cx40 could potentially produce factors that inhibit the formation of these specific vascular defects or provide a nonpermissive environment for their development.

We noted that Cx37 and Cx40 expression occurred in vascular endothelium of only a subset of blood vessels in the E18.5 tissues. Staining of immediately adjacent sections, however, indicated that the same vessels expressed both Cx37 and Cx40 in endothelium. Since endothelial cells are generally elongated cells, it is expected that adjacent sections (10-μm thick) will contain portions of the same endothelial cells. It is therefore highly likely that Cx37 and Cx40 are coexpressed within endothelial cells in the tissues we examined, consistent with prior studies of connexin expression in the vasculature (Yeh et al., 1998; Ko et al., 1999). Previous studies have largely focused on the expression of Cx37 and Cx40 in the arterial system where they are well documented in vessels such as aorta, pulmonary artery, and coronary arteries, but few reports have examined these connexins in the venous system and none have done so in embryonic mouse tissues. Traub et al. (1998) found that Cx37 was not expressed by venous endothelial cells of adult mouse heart or spleen, although Cx37 was present in umbilical cord veins and kidney veins. In addition, in situ hybridization studies with developing chick embryos indicated that the chick ortholog of mouse Cx40 is expressed in the developing arterial vasculature but not in the venous vasculature (Dealy et al., 1994). Our immunostaining results are generally consistent with expression of Cx37 and Cx40 in the arterial system of E18.5 mouse skin, testis, intestine, and lung, and the absence of these connexins in at least portions of the venous vasculature of these tissues.

The phenotype of Cx37−/−Cx40−/− animals is suggestive of a role for Cx37 and Cx40 in normal vascular development. Antibodies against a number of proteins (PECAM-1, Claudin-5, ZO-1, VE-Cadherin, Caveolin-1) expressed by endothelium confirmed that the vascular abnormalities observed in testis and intestine were contained within an extensive endothelium, although some areas had undergone hemorrhage. Vascular defects are classified as either vascular malformations or hemangiomas in part based on the proliferative state of the endothelium. Vascular malformations are characterized by a static endothelium, whereas hemangiomas have an actively proliferating endothelium. It is possible that the absence of Cx37 and Cx40 in testicular blood vessels results in abnormal proliferation of vascular endothelial cells during testicular development, leading to hemangioma formation. Numerous studies have pointed toward a role for gap junction-mediated communication in regulating cell proliferation (Loewenstein and Rose, 1992; Yamashaki et al., 1999). Although we do not yet have direct evidence for increased endothelial proliferation, the elevated expression of PECAM-1 in the endothelial cells of vascular anomalies compared with wild-type vessels suggests that these cells may be in an altered state. Consistent with a developmental role for Cx37 and Cx40, we observed...
vascular defects in Cx37−/− Cx40−/− testis as early as E14.5, before hemorrhages are observed in the animals. Similarly, the enlarged, abnormal vessels observed in Cx37−/− Cx40−/− intestine and skin could also potentially be explained by abnormal proliferation of endothelial cells. These distended vessels might be generally more susceptible to hemorrhage caused by physical stress than normal vessels. During embryonic development, hemodynamic stress steadily increases until birth, and blood vessels are exposed to potential vascular injury. Thus, Cx37−/− Cx40−/− vessels may be more prone to rupture in the late fetal stages and perinatal period. In addition, the absence of endothelial connexins could potentially interfere with normal wound repair mechanisms. It has been proposed that changes in junctional communication are involved in the transition of quiescent endothelial cells into activated migratory or proliferative cells that function during repair of vascular damage (Pepper et al., 1989; Gabriels and Paul, 1998).

Our studies indicate that the absence of Cx37 and Cx40 in endothelial cells does not result in altered cell–cell contact, changes in tight junction protein expression, or increased vascular permeability to large particulates. Silver nitrate staining revealed normal cell–cell interfaces and endothelial cell shape in intestinal vessels. Electron microscopy of intestine and testis further showed the presence of normal cell–cell contacts and persisting intercellular junctions (adherens junctions, tight junctions) between endothelial cells. In addition, expression of tight junction markers (claudin-5, ZO-1, occludin) in vascular endothelium from Cx37−/− Cx40−/− mice appeared normal, suggesting that tight junctions remain intact. Finally, injections of India ink showed that intact Cx37−/− Cx40−/− blood vessels were not abnormally permeable to this particulate tracer. Taken together, these data suggest that, prior to hemorrhage, vascular wall structure is probably normal in Cx37−/− Cx40−/− animals and points more toward a role for Cx37 and Cx40 in vascular development or function. With regard to vascular function, elimination of Cx37 and Cx40 could potentially alter signaling pathways affecting normal blood vessel tone. A role for junctional communication in regulation of vascular tone is supported by recent studies by Chaytor et al. (1998) who showed that gap junction inhibitors block a component of arterial dilatation in rabbits that is NO-independent.

The functional consequences of having multiple connexins expressed in the same cell type are not well understood. Immunofluorescent and immunogold electron microscopy studies have shown that up to three different connexin types can be present in the same endothelial gap junction plaque (Yeh et al., 1998; Ko et al., 1999). Gap junction channels made up of distinct connexin subunits have been shown to display unique channel characteristics in transfected cells and Xenopus oocytes. Multiple connexins could therefore potentially allow for diverse signaling pathways between adjacent cells. Our data, however, suggest at least some functional overlap between Cx37 and Cx40, since vascular abnormalities are only observed when both connexins are eliminated. Since Cx37−/− Cx40−/− animals are viable, a single wild-type allele of Cx37 is sufficient to prevent the vascular phenotype. Recent studies with connexin knock-in mice provide evidence that connexins can have shared as well as unique functions in vivo. In particular, knock-in of Cx32 or Cx40 in place of Cx43 rescued the postnatal lethality associated with Cx43−/− mice, although the knock-in mice differed from wild-type mice in some respects (Plum et al., 2000). It therefore seems likely that Cx37 and Cx40 functionally overlap in vascular endothelial cells, and elimination of both proteins is required to effectively reduce junctional communication to a level that causes the observed vascular phenotype. Our results, however, do not rule out unique roles for these connexins or the possibility that Cx37 and Cx40 could contribute to heteromeric channels with distinct conductances. Furthermore, although changes in endothelial communication are likely to be important for the development of the vascular phenotype, potential changes in myoendothelial or smooth muscle intercellular communication could also contribute. Finally, given the perinatal lethality of the Cx37−/− Cx40−/− phenotype, the development of inducible knockout models would greatly facilitate investigation of the physiological roles of these vascular connexins in mature animals.

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