Vascular Expression of Polycystin-2

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Abstract. The expression of polycystin-1 in the vascular smooth muscle cells (VSMC) of elastic and large distributive arteries suggests that some vascular manifestations of autosomal-dominant polycystic kidney disease (ADPKD) result directly from the genetic defect. Intracranial aneurysms have been reported in PKD2, as well as in PKD1 families. To determine whether the vascular expression of polycystin-2 is similar to that of polycystin-1, the expression of PKD2 mRNA and protein in cultured pig aortic VSMC was studied and immunofluorescence and immunohistochemistry were used to study the localization of polycystin-2 in cultured pig aortic VSMC, pig ascending thoracic aorta, and normal elastic and intracranial arteries and intracranial aneurysms obtained at autopsy from patients without or with ADPKD. Tissues derived from Pkd2 wild-type and Pkd2 null mice were used to confirm the specificity of the immunostaining for polycystin-2. Northern blots of VSMC revealed the expected 5.3-kb band. Western blotting detected a 110-kb band in a 100,000 × g fraction of VSMC homogenates. Cultured VSMC as well as VSMC between the elastic lamellae of pig thoracic aorta were positive for polycystin-2 by immunofluorescence. The staining pattern was cytoplasmic. Treatment of the cells before fixation with Taxol, colchicine, or cytochalasin-D altered the pattern of staining in a way suggesting alignment with the cytoskeleton. The immunohistochemical staining for polycystin-2 was abolished by extraction with 0.5% Triton X-100, indicating that polycystin-2 is not associated with the cytoskeleton. Weak immunoreactivity for polycystin-2, which was markedly enhanced by protease digestion, was detected in formaldehyde-fixed normal human elastic and intracranial arteries. Immunostaining of variable intensity for polycystin-2, which was not consistently enhanced by protease digestion, was seen in the spindle-shaped cells of the wall of the intracranial aneurysms. The similar expression of polycystin-1 and polycystin-2 in the vascular smooth muscle is consistent with the proposed interaction of these proteins in a single pathway. These observations suggest a direct pathogenic role for PKD1 and PKD2 mutations in the vascular complications of ADPKD.

Autosomal-dominant polycystic kidney disease (ADPKD) is a genetically heterogeneous multisystem disorder (1). Mutations in the PKD1 and PKD2 genes are thought to occur in approximately 85 and 15% of patients, respectively, with clinically recognized ADPKD (2). Vascular abnormalities, such as intracranial arterial aneurysms and dolichoectasias and dissections of the thoracic aorta and cervicocephalic arteries, are important extrarenal manifestations of ADPKD (3–13). Intracranial aneurysms occur in type 2 ADPKD, as well as in type 1 ADPKD, although the relative frequency of this complication in the two genotypes is not known (14). The recent observations that polycystin-1, the product of PKD1, is strongly expressed in the smooth muscle cells of normal elastic and large distributive arteries and that its pattern of expression is altered in intracranial aneurysms suggest that some vascular manifestations of ADPKD result directly from the genetic defect (15). Here we report that PKD2 mRNA and polycystin-2 are also expressed in cultured aortic smooth muscle cells and that the pattern of expression of polycystin-2 in normal adult elastic and large distributive arteries and in intracranial aneurysms is similar to that of polycystin-1. The similar expression of polycystin-1 and polycystin-2 in vascular smooth muscle is consistent with the proposed interaction of these proteins in a single pathway and with the occurrence of vascular abnormalities not only in type 1 ADPKD but also in type 2 ADPKD.

Materials and Methods

Aortic Smooth Muscle Cell Cultures

Vascular smooth muscle cells (VSMC) were prepared from pig aorta obtained under sterile conditions (16). The vessels of the aortic arch were ligated, and the aorta was closed at one end. The blood cells and endothelial cells were removed by repeated washing with Earle balanced salt solution (EBSS) containing penicillin and gentamicin and by two incubations with a collagenase solution (0.6 mg/ml) in
EBSS for 15 and 30 min at 37°C. After the collagenase solution was removed from the lumen, the aorta was open aseptically with a scalpel and the layer of intima was carefully shaved off. The remaining tissue was minced and resuspended in a small volume of Dulbecco’s modified Eagle’s medium/F12 media containing 10% fetal bovine serum, and the explants were inoculated into 60-mm dishes with just enough media to cover them. The tissues were refed with fresh media the day after inoculation, one week later, and subsequently, every other day. The cultures were maintained in Dulbecco’s modified Eagle’s medium/F12 media containing 10% fetal bovine serum and used between the third and fifth passages.

Renal Tubular Epithelial Cell Cultures and Transfections

MM55.K cells, a murine renal tubular epithelial cell line, and LLC-PK1 cells, a porcine proximal tubular epithelial cell line, were obtained from American Type Culture Collection. The MM55.K cells were used for Northern analysis, whereas the LLC-PK1 cells were transfected with a full-length PKD2 cDNA expression plasmid and used as positive control in the Western blot experiments. Cells at 70% confluence in 25-cm² flasks were transfected with 8 μg of expression plasmid using a calcium phosphate protocol (17–19). After 16 h, cells were replated into 100-mm dishes and selection with G418 400 μg/ml (Life Technologies, Inc., Gaithersburg, MD) was begun after 48 h in culture. Approximately 3 wk later, resistant colonies were cultured individually and tested for the expression of the full-length PKD2 protein.

Tissue Specimens

Segments of normal pig thoracic aorta were dropped at surgery into a bottle containing cold methyl butane and snap-frozen by immersion into liquid nitrogen. Formaldehyde-fixed, paraffin-embedded specimens obtained at autopsy were used for immunohistochemistry. Control abdominal and thoracic aorta and cervicocephalic arteries were obtained from patients who had autopsies after accidental deaths. Sections of intracranial aneurysms were obtained from five patients with and five patients without ADPKD who had tissue suitable for immunohistochemistry (15). Formaldehyde-fixed, paraffin-embedded cardiac tissues were obtained from E16 Pkd2 wild-type and Pkd2 null mice (20).

Northern Analysis

Total RNA was extracted from aortic smooth muscle cells using the Quick Prep total RNA extraction kit (Pharmacia Biotech, Piscataway, NJ). Twenty μg of total RNA were added to 15 μl of sample buffer (20 μl of 10 × 3-(4-morpholino) propane sulfonic acid, 70 μl of 37% formaldehyde, 200 μl of deionized formamide) and heated to 65°C for 15 min. The entire reaction mixture was loaded into a 1% agarose gel, overlaid with 3-(4-morpholino) propane sulfonic acid buffer, and run for 18 h at 20 to 25 V. After electrophoresis, the gel was soaked in 20 × SSC and the RNA was transferred to a nylon membrane overnight. After transfer, the membrane was rinsed in 2 × SSC and baked in a vacuum for 2 h at 80°C. After prehybridization at 45°C for 4 to 20 h in prehybridization buffer (4.7 ml of 10 × SSPE, 5 ml of formamide, 200 μl of 20% sodium dodecyl sulfate [SDS], 50 μl of

Figure 1. Autoradiograph of Northern blots of mouse proximal tubule epithelium (MM.55.K) and pig aortic smooth muscle (VSM) cell total RNA hybridized with a 32P-radiolabeled PKD2 mouse cDNA probe showing a single 5.3-kb band.

Figure 2. Immunoblots of protein from 100,000 × g fraction of pig aortic smooth muscle cell homogenates stained with YCB9 and YCC2 antiserum. A 110-kD band is seen with both antisera. Each lane contained 6 μg of 100,000 × g pellet protein.
Protein Immunoblotting
Aortic smooth muscle cells plated in T75 flasks were washed in phosphate buffered saline (PBS), scraped, and suspended in ice-cold 250 mM sucrose/1 mM ethyleneglycol-bis(β-aminomethyl ether)-N,N,N',N'-tetraacetic acid/10 mM Hesper-KOH buffer (pH 7.5), containing apro tin (90 μg/ml), benzamidine (1 mM), leupeptin (4 μg/ml), phenyl methylsulfonyl fluoride (0.8 mM), and pepstatin (4 μg/ml). The cells were disrupted with a motor-driven Teflon pestle homogenizer (25 strokes). The homogenates were centrifuged twice at 100,000 × g at 4°C. The resulting supernatants were centrifuged at 100,000 × g for 1 h at 4°C. The membrane was hybridized for 18 h at 45°C in the same buffer containing the random primer 32P-radiolabeled mouse PKD2 cDNA probe or a glyceraldehyde phosphate dehydrogenase probe. The RNA blots were washed with 2 × SSC containing 0.1% SDS for 15 min at room temperature and with 0.2 × SSC containing 1% SDS for 60 min at 55°C. The blots were exposed to x-ray film at −70°C for 20 min using a Kodak intensifying screen (Rochester, NY).

Immunohistochemistry
Tissue sections and aortic smooth muscle cells cultured on Lab-Tek slides, and preimmune sera, active and preadsorbed polycystin-2 antisera, purified polycystin-2 antibodies, rabbit polyclonal antibodies against smooth muscle α-actin, rabbit polyclonal antibodies against smooth muscle-actin (Dako Corp., Carpinteria, CA) and monoclonal mouse antibodies against protein disulfide isomerase (PDI; Affinity BioReagents, Golden, CO) were used for these studies. Tissue sections (4 μm) were deparaffinized in saline, rehydrated in graded ethanol series, and rinsed in tap water. Endogenous peroxidase activity was blocked using 50% methanol/1.5% H2O2. After sections were rinsed in tap water, they were placed in 75 ml of 10 mM citrate buffer (pH 6.0), microwaved for 4 min, allowed to cool for 20 min, and again rinsed in tap water. Sections were then treated with 5% normal goat serum in PBS containing 0.05% Tween 20 for 10 min, incubated at room temperature with the primary antibodies at 1:1000 dilution for 60 min, rinsed once more, and treated with 1:400 biotinylated goat anti-rabbit antibodies (Dako), followed by 1:500 peroxidase-labeled streptavidin (Dako) for 30 min at room temperature. Sections were developed for 15 min by adding 0.1 M sodium acetate (pH 5.2), containing aminoethyl carbazole and H2O2. Counterstaining was carried out with hematoxylin, and coverslips were attached using aqueous mounting media. Limited protease digestions were carried out by applying solutions directly to tissue sections before microwave heating. Proteases used were trypsin (Sigma), 50 mg/ml in PBS (pH 7.5) for 30 min at 37°C, and elastase (type IV; Sigma), 50 μg/ml in PBS (pH 7.5) for 30 min at 37°C. The protocol followed to stain the aortic VSMC grown in Lab-Tek slides was similar to that used to stain the tissue sections without the deparaffinization and microwaving steps. The cells were fixed in cold 10% formaldehyde for 15 min. Limited protease digestions were not performed in the cultured cells.

Immunofluorescence Microscopy
Frozen sections of pig thoracic aorta were cut on a cryostat at 4 to 5 μm, placed on glass slides, and washed three times in PBS. To prevent nonspecific binding of antibodies, we incubated cells and tissue sections for 30 min with 10% goat serum in PBS. Preimmune sera or polyclonal rabbit antisera or affinity-purified antibodies against polycystin-2 were diluted in 10% goat serum in PBS and incubated with the cells for 30 min. After extensive washing with PBS, the cells were incubated again for 30 min with fluorescein-labeled secondary antibodies diluted in 10% goat serum in PBS. Primary and secondary antibodies were used sequentially. The slides were then washed with PBS and mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA), sealed with clear nail varnish, and examined using a Zeiss laser scanning confocal microscope and imaging software.

Results
Northern and Western Analyses
Autoradiographs of Northern blots of pig aortic smooth muscle and mouse proximal tubular epithelial cell total RNA hybridized with a 32P-radiolabeled PKD2 mouse cDNA probe revealed a single 5.3-kb band (Figure 1). Western blotting using either YCC2 or YCB9 antibodies detected a 110-kD band in the 100,000 × g fraction of aortic smooth muscle cell homogenates (Figure 2). Bands of lower molecular weight were less consistently demon-
strated, varied with the total amount of protein loaded, and were deemed to be the result of proteolysis (Figures 2 and 3). The 110-kD band was not seen in Western blots stained with the preimmune sera (Figure 3A), and it was completely competed by the immunizing fusion peptides but not by glutathione-S-transferase (GST) alone (Figure 3B).

Immunohistochemical Staining of Cultured Pig Aortic Smooth Muscle Cells and Cell Treatments

Cultured aortic smooth muscle cells exhibited strong immunohistochemical staining with polycystin-2 antisera and affin-
ity-purified antibodies, as well as with smooth muscle α-actin (Figure 4). No staining was observed with preimmune serum. The staining with the antisera raised against the GST–polycystin-2 fusion proteins was completely blocked by preadsorption with the fusion proteins but was not affected by preadsorption with GST. The pattern of staining observed with the active antisera was fine granular and cytoplasmic.

To determine how cytoskeletal alterations would affect the staining pattern for polycystin-2, we treated aortic smooth muscle cells for 1 to 6 h with Taxol (0.1 μM), colchicine (10 μg/ml), or cytochalasin-D (5 μg/ml) before fixation and immunostaining (Figure 5). Treatment with Taxol for 6 h enhanced the granular pattern of staining with a linear distribution, possibly along microtubular bundles. Treatment with colchicine caused a reduction in size and rounding of the cells with loss of cell–cell contacts and redistribution of the polycystin-2 immunostaining toward the perinuclear region. Treatment with cytochalasin-D also caused a redistribution of polycystin-2 immunostaining to the perinuclear region associated with a reduction in the size of the cells, without loss of cell–cell contacts. These observations indicated that polycystin-2 might be associated to the cytoskeleton or localized in a structure aligned with the cytoskeleton. Despite these marked structural effects, treatment with Taxol, colchicine, and cyto-

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*Figure 7. Effect of elastase digestion on the immunohistochemical staining for polycystin-2 in a human control basilar artery (A through D) and thoracic aorta (E, F). (A) Preimmune serum before elastase digestion. (B) Preimmune serum after elastase digestion. (C) YCC2 antiserum before elastase digestion. (D) YCC2 antiserum after elastase digestion. (E) YCC2 antiserum before elastase digestion. (F) YCC2 antiserum after elastase digestion. Magnification, ×100.*
tochalasin-D for 4 h did not have an effect on the levels of PKD2 mRNA in aortic smooth muscle cells detectable by Northern blotting (Figure 6).

To determine whether polycystin-2 is associated with the cellular cytoskeleton, we exposed pig aortic vascular smooth muscle cells to 0.5% Triton X-100 extraction buffer. This nonionic detergent solubilizes cell membrane and cytosolic proteins but leaves cytoskeletal proteins and proteins associated with the cytoskeleton in the monolayer. The cells were then fixed and stained for polycystin-2. After Triton X-100 extraction, the cytoskeletons and nuclei remained attached to the slide, but no staining for polycystin-2 was detected (Figure 5). This observation suggests that polycystin-2 is contained within a structure aligned with rather than directly associated with the cytoskeleton. The pattern of immunohistochemical staining for PDI, an endoplasmic reticulum marker, was similar to that observed for polycystin-2 (Figure 5).

Immunohistochemical Staining and Immunofluorescence Microscopy of Control Human and Porcine Arteries

Immunostaining for polycystin-2 was detected in all control human arteries (thoracic aortic, internal carotid, vertebral, basilar, posterior communicating, middle cerebral, and anterior communicating arteries). Predigestion of tissue slices with a highly purified preparation of elastase or with trypsin before immunostaining caused disruption of the internal elastic lamina and of the interlaminar elastic fibers evident on the Movat’s stain (not shown, see reference 15). This was accompanied by a marked enhancement in the staining for polycystin-2 (Figure 7). The cells staining for polycystin-2 were located in the media and intima and were also positive for smooth muscle α-actin. The pattern of staining was cytoplasmic. No staining was observed with preimmune sera or with the antiserum raised against GST–polycystin-2 preabsorbed with the fusion protein. Preabsorption with GST alone had no effect.

To confirm the specificity of the immunostaining for polycystin-2, we stained cardiac tissues obtained from E16 Pkd2 wild-type and Pkd2 null mice with our antibodies. Positive staining was detected only in the cardiac myocytes and in the VSMC of the coronary arteries from the wild-type Pkd2 mice. The cardiac myocytes and the VSMC of the Pkd2 null mice, which do not contain polycystin-2, did not stain (Figure 8).

Strong staining for polycystin-2 was also detected in the smooth muscle cells of pig thoracic aorta by immunofluorescence microscopy using the YCB9 and YCC2 antisera. Immunostaining was also observed with the affinity-purified antibodies but not with the preimmune sera. The pattern of staining was cytoplasmic (Figure 9).

Immunohistochemical Staining of Intracranial Aneurysms

By conventional histology, all of the aneurysms selected for this study from five ADPKD patients and five patients without ADPKD had profound structural alterations of their arterial wall with interruption of the internal elastic lamina and of the tunica media at the neck of the aneurysm. The wall of the aneurysm consisted of connective tissue without distinct layers and contained spindle-shaped cells. The smooth muscle α-actin antibodies stained strongly the smooth muscle cells in the media of the parental arteries and to variable extent the spindle-shaped cells in the wall of the aneurysms. Immunostaining of variable intensity for polycystin-2 was seen in the spindle-shaped cells in the wall of the aneurysm as well as in the smooth muscle cells in the media of the parental arteries (Figure 10). Preincubation with elastase did not alter the staining intensity in the wall of the aneurysms. The pattern of polycystin-2 immunostaining in the aneurysms from patients with and without ADPKD was not noticeably different.

Discussion

The studies presented here demonstrate that PKD2 mRNA and polycystin-2 are strongly expressed in the smooth muscle

Figure 8. Immunohistochemical staining for polycystin-2 of cardiac tissue obtained from E16 Pkd2 wild-type (A) and Pkd2 null (B) mice, using YCC2 antiserum. Note the positive staining of the cardiac myocytes and vascular smooth muscle cells (VSMC) of a small coronary in the wild-type mouse. No immunostaining was detected in the Pkd2 null mouse. Magnification, ×400.

Figure 9. Indirect immunofluorescence microscopy of pig thoracic aorta using preimmune serum (A) and YCB9 antiserum (B). Note the staining of the VSMC between the elastic lamellae with the YCB9 antiserum but not with the preimmune serum. The pattern of staining is cytoplasmic. Magnification, ×250.
cells of elastic arteries. This observation is consistent with the recent description of intracranial aneurysms in patients with type 2 ADPKD (14) and favor the hypothesis that genetically determined alterations in the expression of polycystin-2 are directly responsible for the predisposition of some patients with type 2 ADPKD to develop intracranial aneurysms and possibly other vascular abnormalities.

Another finding of this study is that the pattern of expression of polycystin-2 in normal elastic arteries and intracranial aneurysms is identical to that previously described for polycystin-1 (15). This observation is not unexpected, because it has been reported that polycystin-1 and polycystin-2 interact at their carboxyl terminal portions, suggesting that they may be interacting partners of a common pathway (22,23). Further support for the hypothesis is provided by the recent observations that polycystin-1 is required for the structural integrity of the blood vessels and that mouse embryo homozygous for a mutant Pkd1 or for a Pkd2 null allele exhibits a lethal phenotype characterized by diffuse vascular ruptures and hemorrhage (20,24).

The enhancement of immunostaining for polycystin after partial elastase or trypsin digestion in normal arteries of control subjects without ADPKD was also observed for polycystin-1 (15). The significance of this observation is not certain, but it may suggest that polycystin-1 and polycystin-2 are part of supramolecular arrangements. In conventional formaldehyde-paraffin sections, antigens may become masked because of the formation of methylene bridges between reactive sites on the same molecule or adjacent proteins (25). The masking of the antigenic determinants may be particularly prominent for densely packed proteins because their proximity facilitates strong intermolecular cross-linkages (26). The significance of the increased immunoreactivity for polycystin-2 and the lesser susceptibility to protease digestion noted in the intracranial aneurysms is also uncertain. Nevertheless, differential susceptibility to protease digestion has been noted for other proteins in other pathologic conditions (27).

Recently, we reported that in cultured untransfected or PKD2 transfected tubular epithelial cells, polycystin-2 is localized within the endoplasmic reticulum and perhaps the cis-Golgi (19). In these cells, polycystin-2 was found to be membrane bound, N-glycosylated, and completely sensitive to endoglycosydase H digestion, a characteristic of proteins retained in the endoplasmic reticulum and cis-Golgi. Furthermore, polycystin-2 was found to co-localize with endoplasmic reticulum markers by subcellular fractionation using linear density gradient centrifugation and by double immunofluorescence. Finally, the retention of polycystin-2 in the endoplasmic reticulum was found to be dependent on the presence of the carboxyl terminus domain (19).

Figure 10. Immunohistochemical staining for polycystin-2 in an intracranial aneurysm. (A) YCC2 antiserum before elastase digestion. (B) YCC2 antiserum after elastase digestion; note disruption of the internal elastic lamina at the neck of the aneurysm (arrow). (C) Preimmune serum. (D) YCC2 antiserum without elastase digestion; the arrowheads point to the internal elastic lamina. Magnifications: ×100 in A and B; ×250 in C and D.
Predictions based on the amino acid sequence of polycystin-1 and polycystin-2 suggest that these are integral membrane proteins that contain several intramembranous domains (28–31). The subcellular localization of polycystin-2 in the present study seemed to be mainly cytoplasmic and membrane associated and therefore consistent with the localization in the endoplasmic reticulum noted in cultured tubular epithelial cells in our previous study (19). The pattern of immunostaining for polycystin-2 in VSMC was affected by the treatment with Taxol, colchicine, and cytochalasin-D. Taxol promotes assembled aggregation of excessively stable microtubules (32,33). Colchicine and cytochalasin-D promote the dissociation of microtubules and actin filaments by preventing the polymerization of tubulin and actin, respectively (34,35). The redistribution of the polycystin-2 immunostaining after treatment with these drugs could suggest an association of polycystin-2 with the cytoskeleton. However, the disappearance of polycystin-2 immunoreactivity after extraction with a nonionic detergent indicates that polycystin-2 is localized in membrane-bound structures that are aligned with the cytoskeleton. These observations are also consistent with a localization of polycystin-2 in the endoplasmic reticulum. Nevertheless, they do not rule out the possibility that some polycystin-2 may also be located in the plasma membrane of VSMC.

This study was not designed to assess the function of polycystin-2. Therefore, it is possible to speculate only on the functional significance of the localization of polycystin-2 in the endoplasmic reticulum of VSMC. Recent observations indicate that protein complexes or aggregates of large molecular size within a reticular-like matrix in the endoplasmic reticulum are important for the normal maturation of collagens and other extracellular matrix proteins (36). The localization and staining characteristics of polycystin-2 described in the current study, as well as the nature of the extrarenal manifestations of AD-PKD, are consistent with a role for polycystin-2 in the processing of structural cellular or secreted extracellular proteins.

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References