Effects of Angiogenic Factor Overexpression by Human and Rodent Cholangiocytes in Polycystic Liver Diseases

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Liver involvement in autosomal dominant polycystic kidney disease (ADPKD) is characterized by altered remodeling of the embryonic ductal plate (DP) with presence of biliary cysts and aberrant portal vasculature. The genetic defect causing ADPKD has been identified, but mechanisms of liver cyst growth remain uncertain. To investigate the possible role of angiogenic mechanisms, we have studied the immunohistochemical expression of vascular endothelial growth factor (VEGF), angiopoietin–1 (Ang-1), angiopoietin-2 (Ang-2) and their receptors (VEGFR-1, VEGFR-2, Tie-2) in ADPKD, Caroli's disease, normal and fetal livers. In ADPKD and control livers Ang-1 and Ang-2 gene expression was studied by real-time-PCR. Effects of VEGF on cholangiocyte proliferation were studied by PCNA Western Blot in isolated rat cholangiocytes and by MTS assay in cultured cholangiocytes isolated from ADPKD patients and from an ADPKD mouse model ($Pkd2^{WS25/-}$). Cholangiocytes were strongly positive for VEGF, VEGFR-1, VEGFR-2 and Ang-2 in ADPKD and Caroli, and also for Ang-1 and Tie-2 in ADPKD, similar to fetal ductal plate cells. VEGF stimulated proliferation in both normal and ADPKD cholangiocytes, but the effect was particularly evident in the latter. Ang-1 alone had no effect, but was synergic to VEGF. VEGF expression on cholangiocytes positively correlated with microvascular density. In conclusion, consistent with the immature phenotype of the cystic epithelium, expression of VEGF, VEGFRs, Ang-1 and Tie-2 is strongly upregulated in cholangiocytes from polycystic liver diseases. VEGF and Ang-1 have autocrine proliferative effect on cholangiocyte growth and paracrine effect on portal vasculature, thus promoting the growth of the cysts and their vascular supply. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006;43:1001-1012.)

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; Ang-1, angiopoietin 1; Ang-2, angiopoietin 2; BEC, biliary epithelial cells; DPM, ductal plate malformation; sDP, single layer ductal plate; dDP, double layer ductal plate; iBD, incorporating bile duct; BD, incorporated bile duct; EC, endothelial cell; GW, gestational weeks; HRP, horseradish peroxidase; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MVD, microvascular density; NCAM, neural cell adhesion molecule; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor, WT, wild type.

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Received April 25, 2005; accepted January 29, 2006.

Financial support of Telethon (E.1253) to L.F., Ministero dell'Università e della Ricerca Scientifica e Tecnologica (grant 2003060498_001) to L.O., Associazione Mary Papagni-Cefis and Fondazione S. Martino, Bergamo to MS and of MS recruiting grant from Yale University and Yale Liver Center (DKP 30/36989) are gratefully acknowledged.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21143

Potential conflict of interest: Nothing to report.

See Editorial on Page 906

Polycystic liver diseases represent important examples of genetic cholangiopathies; these are diseases of the intrahepatic biliary tree that lead to various degrees of chronic liver impairment and to a variety of complications, including cholangiocarcinoma. Among polycystic liver diseases, autosomal dominant polycystic kidney disease (ADPKD) is characterized by an abnormal development of both renal tubular and intrahepatic biliary epithelia, which undergo a progressive cystic enlargement causing renal insufficiency and massive hepatomegaly with mass effect or, more rarely, to cyst hemorrhage, infection, or rupture. ADPKD is due to mutations in the genes encoding for polycystin-1 or -2, membrane protein complexes localized at focal adhesions, cell-cell junctions and cilia. Polycystins are believed to act as membrane mechanoreceptors able to translate signals from the extracellular environment into transcriptional regulations of proteins that control epithelial cell differentiation and maturation.[1]

In the liver, the abnormal development of the small intrahepatic bile ducts leads to the formation of multiple biliary microhamartomas that progressively dilate to become macroscopic cysts. No medical treatment is currently available to prevent the progressive growth of these biliary cysts. The fetal configuration of biliary microhamartomas suggests a failure in the physiological remodeling of the embryonic ductal plate, hence the definition of ductal plate malformation (DPM).[2] Often associated with DPM is an abnormal ramification pattern of the portal vasculature in close vicinity to cysts and microhamartomas ("pollard willow" pattern).[3]

Endothelial and epithelial cells are known to exchange multiple paracrine signals,[4] particularly during morphogenesis and tissue remodeling.[5,6] A variety of different signals and, in particular, angiogenic growth factors and their receptors is involved in the regulation of the vascular growth and differentiation. The vascular endothelial growth factor (VEGF) and receptor (VEGFR) system and the angiopoietins/Tie-2 system are key regulators of the embryonic vascular development. VEGF interacts with two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), expressed by the vascular endothelial cells (EC), promoting their differentiation and proliferation.[7] Although originally thought to be restricted to vascular cells, VEGFR-1 has been recently shown to be expressed by epithelial cells during development, as seen for glomerular and tubular cells in fetal rat kidneys.[5] Angiopoietins, namely angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), are a second family of vascular growth factors, acting in concert with VEGF to promote the remodeling, maturation, and stabilization of blood vessels. Angiopoietins bind to the Tie-2 receptor, a tyrosine kinase expressed by ECs, together with VEGF receptors.[8] The two angiopoietins have opposite effects on Tie-2: Ang-1 activates Tie-2 by inducing its tyrosine phosphorylation, while Ang-2 antagonizes the Ang-1/Tie-2 binding.[9] Therefore, the level of Tie-2 activation is determined by the relative balance between Ang-1 and Ang-2.

There is much evidence to indicate the involvement of angiogenic growth factors in the pathogenesis of developmental diseases affecting kidney and lung epithelia.[5,10] Given the histological evidence of the existence of biliary and vascular abnormalities in ADPKD livers, it was our hypothesis that local release of angiogenic factors may be involved in the abnormal cystic development of bile ducts. We have thus studied the expression profile of VEGF and angiopoietins in ADPKD and in a genetically distinct DPM-related cystic cholangiopathy, such as Caroli's disease, as well as in fetal livers.

Materials and Methods

Liver Tissue

Diseased liver tissue (ADPKD n = 21, Caroli's disease n = 8) was obtained from liver transplants performed in three different European centers (Bergamo, Italy; University-Hospital of Birmingham, UK; University of Leuven, Belgium). Needle biopsies were also obtained from patients with minimal abnormalities of liver function tests, but normal histology (absence of bile duct damage or loss, ductular proliferation, steatosis, necrosis, or hepatitis) (n = 4), and served as control livers. All diagnoses were based on clinical and laboratory data and on histopathological examination of routinely processed tissue. Fetal liver was taken at autopsy of 23 fetuses aged from 8 to 40 gestational weeks (GW) performed in Leuven. Liver tissue was snap-frozen in liquid-nitrogen cooled isopentane and stored at −70°C. Informed consent and local regional ethical committee approval were obtained before tissue collection.

Immunohistochemistry

Peroxidase and Double Immunofluorescence Staining. Acetone-fixed 4μm serially cut freshly frozen tissue sections were immunostained as described[11] to visualize the localization of the following primary antibodies (see Table 1 for details) directed against VEGF (which recognizes $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ splicing variants), VEGFR-1, VEGFR-2, Ang-1, Ang-2 and Tie-2. Cell distribution of angiogenic factors was then compared to both anti-HEA-125 and anti-CD31 (PECAM-1) antibodies to allow a correct identification of biliary epithelial cells (BEC) and vascular ECs, respectively. To study co-ex-

Table 1. Primary Antibodies Used for Immunohistochemistry and Biological Significance of the Corresponding Markers

Markers	Source	Isotype	Diluition	Biological Significance
HEA-125	Progen Biotechnik Gmbh	IgG_1 (mouse)	1:100 (HRP) 1:100 (IF)	34-kd epithelial surface glycoprotein (egp34) biliary lineage-specific homologous to nidogen
CD31 (PECAM-1)	NeoMarkers	IgG_1 (mouse)	1:40 (HRP) 1:20 (IF)	Glycoprotein involved in cell adhesion and cell signaling, selectively expressed by EC (even sinusoidal EC)
CD34	Novocastra	IgG_1 (mouse)	1:25 (HRP)	110-kd monomeric cell surface antigen, selectively expressed by EC
VEGF	Santa Cruz Inc.	Rabbit polyclonal	1:50 (HRP) 1:20 (IF)	Dimeric glycoprotein involved in angiogenesis and tumorigenesis, exerting mitogenic activity on EC
VEGFR-1 (Flt-1)	Santa Cruz Inc.	Rabbit polyclonal	1:50 (HRP) 1:20 (IF)	Cell membrane tyrosine-kinase receptor for VEGF, involved in vessel branching morphogenesis
VEGFR-2 (Flk-1 or KDR)	Santa Cruz Inc.	IgG_1 (mouse)	1:100 (HRP) 1:20 (IF)	Cell membrane tyrosine-kinase receptor with high affinity for VEGF, involved in ECs mithogenesis and migration
Ang-1	Santa Cruz Inc.	Goat polyclonal	1:75 (HRP) 1:20 (IF)	Ligand for Tie-2, involved in EC development
Ang-2	Santa Cruz Inc.	Goat polyclonal	1:75 (HRP) 1:20 (IF)	Ligand for Tie-2, where it antagonizes Tie-2 activation by Ang-1; expressed by vessels undergoing regression
Tie-2	Santa Cruz Inc.	Rabbit polyclonal	1:50 (HRP) 1:20 (IF)	EC surface tyrosine-kinase receptor for Ang-1 and Ang-2 involved during embryonic vascular development and tumorigenesis

NOTE. In IF the following secondary antibodies were used: FITC-conjugated anti-rabbit (Santa Cruz Inc., goat anti-rabbit clone sc-2012, dilution 1:20), anti-goat (DAKO, rabbit anti-goat, dilution 1:20) and anti-mouse (DAKO, rabbit anti-mouse, dilution 1:20), and Texas Red-conjugated anti-mouse (Vector, horse anti-mouse, dilution 1:20). Dual-immunofluorescence has been performed according to us.[2]

Abbreviations: HRP, horseradish peroxidase; IF, immunofluorescence.

pression of angiogenic factors on ductular and vascular structures double immunostaining was performed in selected cryostat sections, as detailed in Table 1 and in the online section (available at the HEPATOLOGY website, http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).

Histopathological Evaluation

DPM-Related Diseased Liver. *Biliary microhamartoma* was defined as a cluster of irregularly shaped, dilated bile ducts, lined by a layer of cuboidal, HEA-125-positive BEC, while *biliary cyst* was defined as a grossly dilated bile duct, generally accompanied by multiple biliary microhamartomas, often containing intraluminal bile, and lined by cuboidal or flattened BEC. *Arteries* and *veins* were identified by CD31 expression on EC, being arteries surrounded by a well-recognizable muscular wall.

Fetal Liver. Developing biliary structures were categorized according to Libbrecht et al.[12] as reported in the legend to Fig. 5.

Histological Assessment. Stained tissue sections were assessed by two independent observers (L.F, A.S.). In diseased liver tissue, the proportion of biliary structures which were positive for each angiogenic factor was semiquantitatively scored,[12] as 0 (negative staining or weak signal not clearly emerging from the background), 1 (<20% of structures positive), 2 (from 20% to 70%) and 3 (>70%). The proportion of samples with high-grade staining (score 2 and 3) of biliary structures (normal bile ducts, or biliary microhamartomas and biliary cysts in developmental cholangiopathies) was then calculated for each angiogenic factor. In fetal tissue, angiogenic factor staining was assessed in developing biliary epithelium and vascular endothelium as negative (absence of staining), weak (positive staining restricted to few cells), or positive (positive staining clearly observed in the majority of cells).

Morphometric Analysis of VEGF-Positive Bile Duct Area and Microvascular Density (MVD). In digital images of 3 non-overlapping random fields taken at 200× from ADPKD (n = 10) and Caroli's disease (n = 4), the VEGF-positive area of biliary structures was calculated using the UTHSCSA Image Tool 3.0 software (University of Texas, San Antonio, TX), as the percentage of pixels above the threshold value with respect to the total pixels per field. In homologous fields of serial sections stained for CD34 (an EC marker), we also determined the number of microvessels in the portal space as number of CD34-positive structures.[13]

Real Time-PCR Analysis

Total RNA was extracted from liver samples obtained from ADPKD (n = 16) and control livers (n = 4) according to the guanidinium thiocyanate method[14] using the Tri Reagent solution (Sigma Chemical Co., Milan, Italy). Details on the PCR method can be found online at the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).

Proliferating Cell Nuclear Antigen (PCNA) Analysis by Western Blot in Isolated Rat Cholangiocytes. Cholangiocyte proliferation was assessed by measuring PCNA protein expression (Western Blot) as described,[15]

in cholangiocytes isolated from Sprague Dawley rats (details online at http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).

Cholangiocyte Isolation and Culture From a Mouse Model of ADPKD. The $Pkd2^{WS25/-}$ mouse, kindly provided by Dr. S. Somlo (Yale University, New Haven, CT), is orthologous to human ADPKD due to PKD2 deficiency.[16] This mouse consistently develops both the kidney and liver phenotype of ADPKD. Wild-type (WT) mice were used as control. WT and cystic cholangiocytes were isolated by microdissection of the portal tissue following a modification of the method of Masyuk et al.[17] Microdissected ducts and cysts were plated inside collagen gel as described for microdissected polycystic kidney tubules by Nishio et al.[18] and then cultured on an enriched medium as described,[19] slightly modified by increasing fetal bovine serum (FBS) to 10%. Cholangiocytes outgrew and eventually formed a monolayer. Biliary phenotype of cultured cells was confirmed by positive cytokeratin 7 immunostaining.

Human Cholangiocyte Isolation and Culture

Human cholangiocytes were purified from liver tissue obtained from the liver transplantation program in Bergamo as described.[20] One patient with ADPKD was transplanted for massive liver enlargement with mass effect; cholangiocytes, isolated from a patient transplanted for alcoholic cirrhosis, served as controls.

Determination of Cell Proliferation by MTS

Human and mouse cholangiocytes maintained in 25 cm^2 tissue culture flasks in medium enriched with 3% and 10% FBS, respectively, at 37°C in a humidified, 5% CO_2 atmosphere, were passaged and then cultured in a 96-multiwells plate (5000 cells/well) in a quiescent medium (0.5% FBS). After 48 hours, cells were supplemented with human or mouse recombinant VEGF165 and/or Ang-1 (R&D Systems, Space, Milan, Italy) at different concentrations for 4 days, as shown in the result section. Proliferation was assessed by the CellTiter 96 AQueous One Solution (Promega Italia, Milan, Italy), which exploits the MTS tetrazolium compound colorimetric bioreduction by the cells.

Measurement of VEGF Levels

Enzyme-linked immunosorbent assay (ELISA) for VEGF (Biosource International Inc., Camarillo CA) was performed to quantify the amount of this factor in the culture medium collected from cholangiocytes isolated from ADPKD and livers with alcoholic cirrhosis (5000 cells/well; 96 well plate). For linearity cell culture media was diluted at 1:2.

Statistical Analysis

Results are shown as mean ± SD. Statistical analysis of immunohistochemical data was performed using the Chi-squared test, while statistical comparisons of gene expression data were made using Student's *t* test. Correlation between the VEGF-positive ductular area and MVD was calculated using the Spearman test. Statistical analysis was performed using the SPSS software 13.0 (SPSS Inc., Bologna, Italy), and *P* values less than .05 were considered as significant.

Results

Immunohistochemistry

Normal and Polycystic Liver Diseases. We have compared the localization of VEGF, angiopoietins and their respective receptors in ADPKD, Caroli's disease and control livers. Results are shown in Figs. 1 and 2. As seen by dual immunofluorescence with CD31, no differences in the expression pattern of angiogenic factors and receptors on the vascular structures could be observed between diseased and control livers (data not shown), while on the biliary epithelium the expression of angiogenic factors was markedly increased in ADPKD and Caroli's disease with respect to control livers.

VEGF and VEGF Receptors. In controls, high-grade expression of VEGF and VEGFR-1 on bile ducts, regardless of their size, was never observed; grade 2 expression of VEGFR-2 was seen in one case. A weak, diffuse VEGF expression was detectable on hepatocytes, localized mainly at the cell membrane (Fig. 1A-C). In biliary cysts from ADPKD samples, high-grade staining was commonly observed for both VEGF (14/17 of the cases with recognisable cysts, 82.3%) and its receptors, VEGFR-1 (11/14, 78.6%) and VEGFR-2 (13/15, 86.6%) (Fig. 1D-F). Similarly, high-grade staining was present in Caroli's disease for VEGF (4/5 specimens, 80%), VEGFR-1 (4/5, 80%) and VEGFR-2 (4/4 specimens, 100%) (Fig. 1G-I). No immunophenotypic differences between biliary cysts and biliary microhamartomas could be detected in both ADPKD and Caroli's disease.

Angiopoietins and Tie-2. In normal bile ducts, neither Ang-1 nor Tie-2 showed high-grade expression (0/3), while grade 2 Ang-2 expression was seen in one case; a diffuse, homogeneous Ang-1 positive staining was present in hepatocytes (Fig. 2A-C). In ADPKD high-grade staining was commonly seen in biliary structures for Ang-1 (12/14 of cases, 85.7%), Ang-2 (14/16, 87.5%) and Tie-2 (12/14, 85.7%) (Fig. 2D-F). In Caroli's disease, while biliary cyst and microhamartoma Ang-2 expression was comparable to ADPKD (3/4, 75%), a remarkable difference was present in Ang-1 (1/4, 25%; $\chi^2 = 5.2191$, $P < .05$) and Tie-2

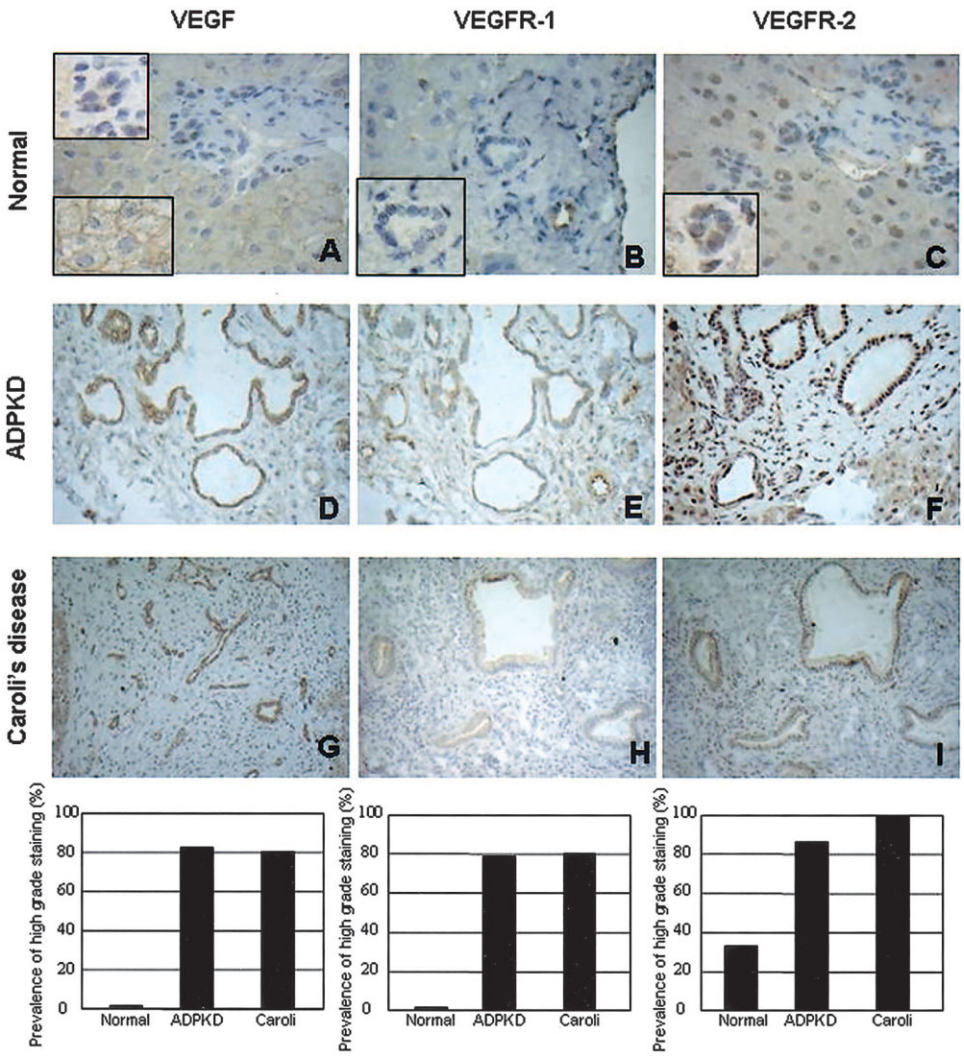

Fig. 1. Immunolocalization and distribution of staining intensity of VEGF, VEGFR-1 and VEGFR-2 in normal and diseased liver tissue. In normal liver VEGF (A), VEGFR-1 (B) and VEGFR-2 (C) staining is negative or rarely positive in bile ducts. In contrast, VEGF, VEGFR-1 and VEGFR-2 expression on bile duct epithelium is strongly upregulated in both ADPKD (D-F) and Caroli's disease (G-I) without any significant difference. A diffuse VEGFR-2 immunoreactivity in hepatocytes was observed in the two cholangiopathies but not in controls. In vascular structures VEGF was negative, while VEGFR-1 and VEGFR-2 were positive in ECs in both arteries and veins and their intensity did not differ between controls and the diseased livers. Original magnifications: G-I ×200, D-F ×400, A-C ×600; figure insets (A-C): ×1000. The column plot below sided shows the prevalence of high-grade staining for VEGF, VEGFR-1 and VEGFR-2 in normal and cystic bile ducts.

(2/7, 28.6%; $\chi^2 = 6.8571$, $P < .01$) (Fig. 2G-I) expression that was significantly lower with respect to ADPKD. In both ADPKD and Caroli's disease, biliary cyst immunophenotype was not different from that of microhamartomas. The expression profile of the angiopoietins and Tie-2 in portal arteries and veins did not differ between controls and DPM-related cystic diseases: Ang-2 and Tie-2 were expressed on EC and supporting cells, while Ang-1 was negative in vascular structures.

To confirm that angiogenic growth factors were indeed localized to the biliary epithelium, including its most immature structure (such as the ductal plate remnant), dual-immunofluorescence staining was performed by matching the different angiogenic markers with HEA-125 (BEC marker) and CD31 (EC marker). Co-expression of the biliary marker HEA-125 and angiogenic factors/receptors could be detected in microhamartomas and cysts in ADPKD, while co-localization of Ang-1 and Tie-2 with HEA-125 was rarely seen in Caroli's disease (Fig. 3A-F).

Correlation Between VEGF Expression on Biliary Structures and Microvascular Density in ADPKD and Caroli's Disease. As shown in Fig. 4, we found a strong, linear correlation between the ductular area expressing VEGF and MVD ($r_s = 0.61$, $P < .01$), consistent with the hypothesis that VEGF produced by cholangiocytes may promote, in paracrine fashion, an expansion of the portal vascular bed surrounding the abnormal biliary structures.

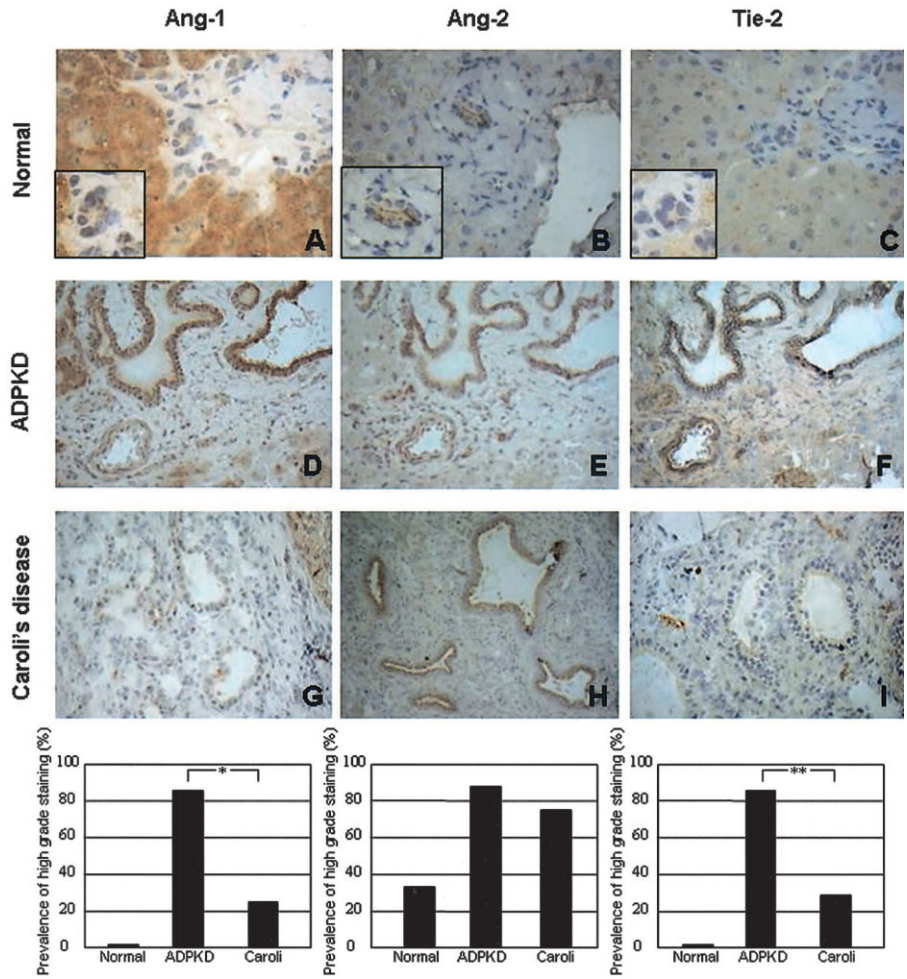

Fig. 2. Immunolocalization and distribution of staining intensity of Ang-1, Ang-2 and Tie-2 in normal and diseased liver tissue. In normal liver Ang-1 (A), Ang-2 (B) and Tie-2 (C) staining is absent or rarely positive in bile ducts. In ADPKD strong over-expression of Ang-1 (D), Ang-2 (E) and Tie-2 (F) is seen in biliary structures. In Caroli's disease Ang-2 (H) shows a similar grade of expression on biliary structures, while significantly lower levels were observed for Ang-1 (G) and Tie-2 (I). Original magnifications: D-G ×400, A-C and I ×600, H ×200; figure insets (A-C) ×1000. The column plot below sided shows the prevalence of high-grade staining for Ang-1, Ang-2 and Tie-2 in normal and cystic bile ducts. Statistical significance (*$P <$ .05, **$P <$ .01) calculated by using the chi-squared test.

Fetal Liver. Immunostainings were performed in fetal liver from different gestational ages (8-40 GW). Results are shown in Fig. 5. Notably, angiogenic factors and receptors were not restricted to endothelium, but they were also expressed by the developing biliary epithelium, in a manner depending on the maturation stage of the biliary structure (see diagram in Fig. 5).

VEGF and VEGF Receptors (Fig. 5A-F) . Biliary cells showed a strong and persistent VEGF immunoreactivity throughout the different developmental stages, from the most immature single ductal plate (sDP) to the more differentiated bile ducts (BD) (Fig. 5A-B), despite a persistent VEGF negativity in vessels. Conversely, VEGFR-1 immunoreactivity was present in vessels and in biliary cells, decreasing progressively in intensity as bile structure maturation proceeded from sDP to double ductal plate (dDP) (Fig. 5C-D). VEGFR-1 expression on EC was more pronounced in developing arteries than in veins. A faint VEGFR-2 positivity could be observed only in sDP and in scattered hepatoblasts, being downregulated soon afterwards; vessels and more mature BD were both negative for VEGFR-2 (Fig. 5E-F).

Angiopoietins and Tie-2 (Fig. 5G-H). Throughout the biliary epithelium development from DP to mature BD, biliary cells did not express Ang-1 or Ang-2 (not shown). In hepatoblasts Ang-1 was constantly positive, while Ang-2 was negative. In vessels, Ang-2 was expressed by EC, while Ang-1 was negative. On the other hand, Tie-2 immunoreactivity was intense in biliary cells during the DP stage, and decreased during the following stages, becoming faint and patchy in BD (Fig. 5G-H). Tie-2 was positive also in vessels, both in EC and in surrounding support cells.

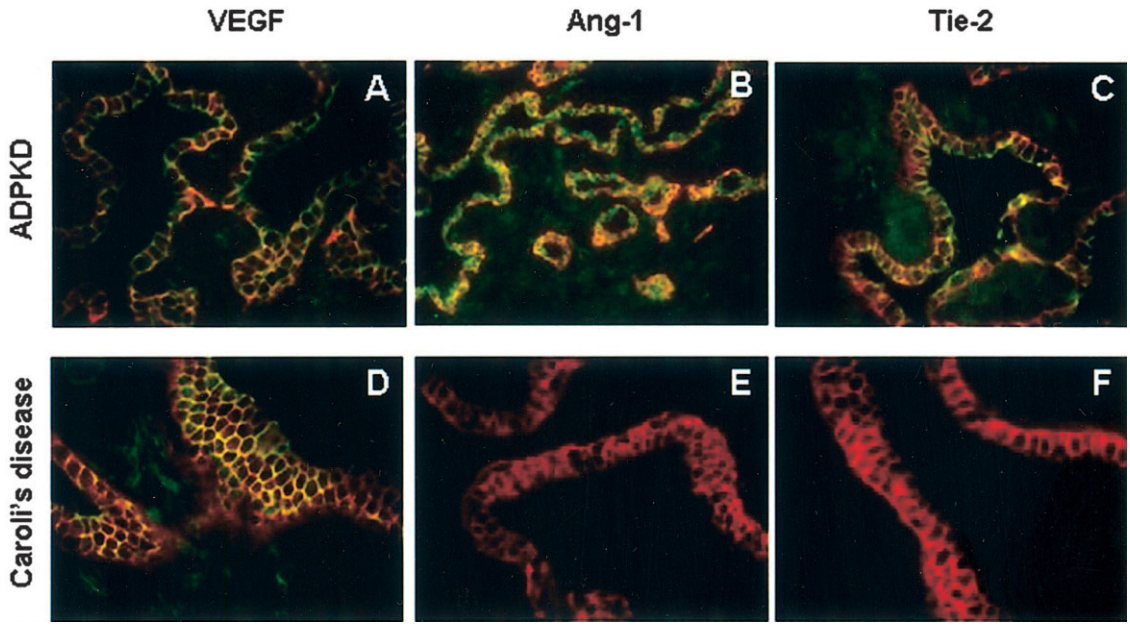

Fig. 3. Dual-immunofluorescence staining of liver sections from ADPKD and Caroli's disease highlights the major phenotypic differences between the two cystic cholangiopathies. In ADPKD double staining of the biliary cell marker HEA-125 (red fluorescence) with VEGF (green fluorescence, A), Ang-1 (green fluorescence, B) and Tie-2 (green fluorescence, C) confirms that these angiogenic factors strongly co-localize (yellow fluorescence) to bile duct epithelium. In Caroli's disease double staining of HEA-125 (red fluorescence) with VEGF (green fluorescence, D), Ang-1 (green fluorescence, E) and Tie-2 (green fluorescence, F) reveals co-localization (yellow fluorescence) to cholangiocytes for VEGF only. Original magnifications: A and C-F: ×600, B ×400.

Gene Expression of Ang-1 and Ang-2 in Controls and ADPKD

Because the net effect on Tie-2 depends on the competition between the agonist and antagonist action of Ang-1 and Ang-2 respectively, we have quantified their gene expression by real time-PCR according to Paradis[21] and expressed as ratio. As reported in Fig. 6, the Ang-1/Ang-2 mRNA ratio was shown to be increased in 9/16 (56%) ADPKD samples with respect to the highest normal value, thus implying an overall activating effect of Ang-1 on Tie-2.

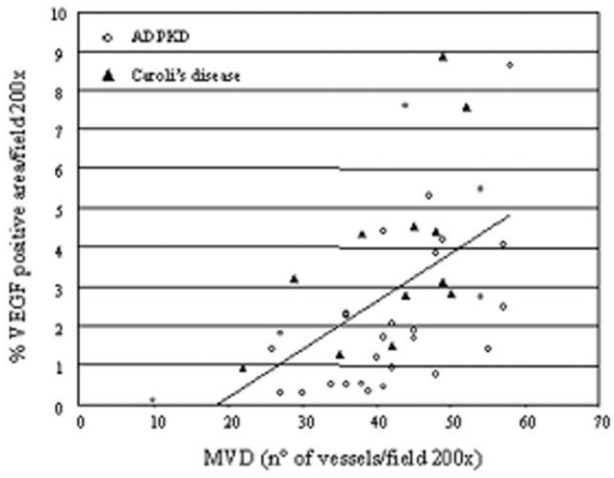

Fig. 4. Correlation between VEGF-positive area on biliary structures and the MVD. VEGF-positive ductular area closely correlated with the extent of portal vascularization in ADPKD and Caroli's disease ($r_s = 0.61$, $P < .01$).

VEGF Secretion in Cultured Human Cholangiocytes

Using human cholangiocytes isolated from ADPKD and from alcoholic cirrhosis (control), we measured the concentration of VEGF on collected culture medium, to verify whether cholangiocytes were actually capable to substantial secretion of VEGF. Our results show cholangiocytes do secrete VEGF in the medium and that VEGF concentration is significantly higher (326 ± 134 pg/mL; n = 9) in the culture medium of cholangiocytes isolated from ADPKD livers, than in that of control cholangiocytes (109 ± 66 pg/mL; n = 9, $P < .001$).

Effect of VEGF and Angiopoietins on Cholangiocyte Proliferation

It is possible that VEGF and angiopoietins exert a proliferative effect on epithelial cells as in ECs.[22,23] Since VEGF receptors and Tie-2 are expressed by normal rat cholangiocytes (not shown), as also reported for VEGFR-2 by others,[24] we investigated the proliferative action of VEGF, Ang-1 and Ang-2 on rat cholangiocytes, by measuring their effects on PCNA protein expression. VEGF significantly increased PCNA protein expression in a dose-dependent way, while Ang-1 and Ang-2 had

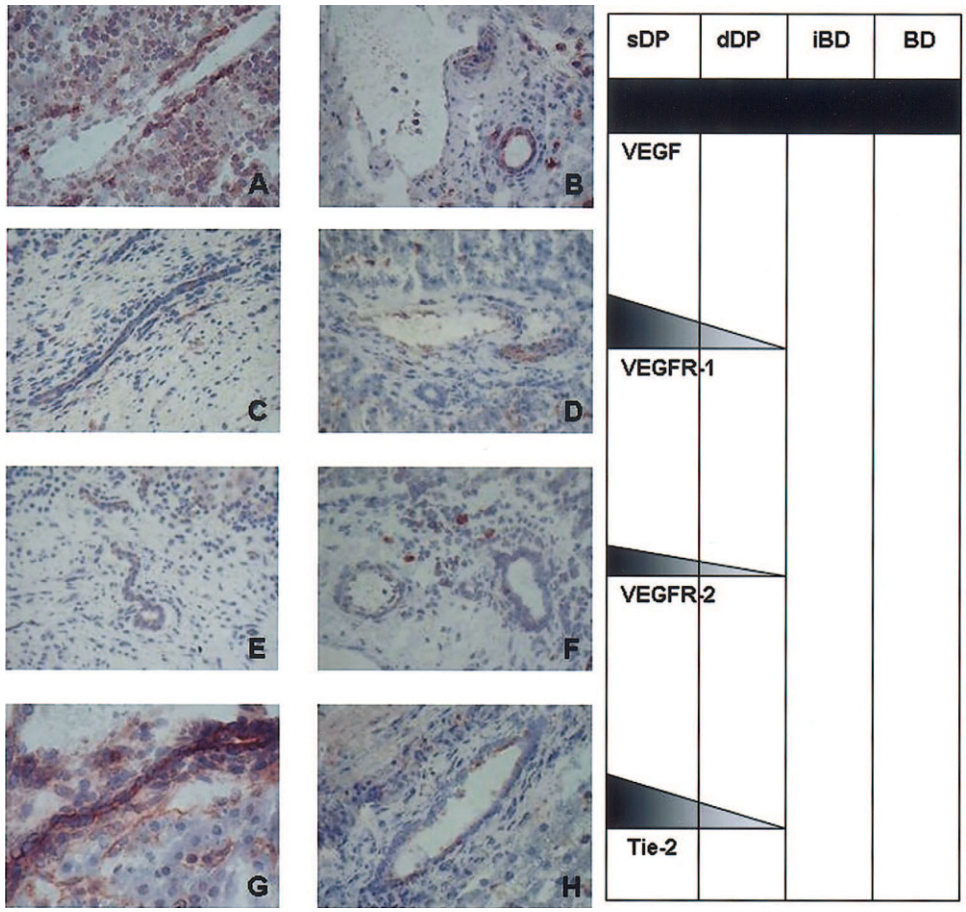

Fig. 5. Immunolocalization of VEGF (A-B), VEGFR-1 (C-D), VEGFR-2 (E-F) and Tie-2 (G-H) during embryonic development of biliary epithelium. On the right a schematic diagram showing the expression intensity changes of these angiogenic factors during the different developmental stages of bile duct ontogeny. A ductal plate (DP) was defined as a sheath of cuboidal cells circularly located around a portal vein branch at the parenchymal-mesenchymal interface. The different maturation stages of the early DP were categorized as single layer (sDP, *i.e.*, the most immature recognizable biliary structure) or as double layer (dDP, duplicated layer over variably long segments of its perimeter). At later developmental stages, tubular segments derived from the dDP begin to be incorporated into the portal mesenchyme as individualized bile ducts (incorporating bile ducts, iBD), which then become fully incorporated bile ducts (BD), being completely surrounded by a thick mesenchymal tissue. VEGF is early expressed by developing ductal plates and then maintained until advanced maturation stages. The angiogenic receptors, VEGFR-1, VEGFR-2 and Tie-2, are early expressed in the ductal plate stage, but gradually disappear afterwards. Original magnifications: A-H ×400.

only little or no effect (Fig. 7A). However Ang-1, when administered together with VEGF (25ng/mL),[25] a strong stimulation of PCNA expression was noticed. On the other hand, Ang-2, added at increasing concentrations to a mixture of VEGF plus Ang-1, was capable to inhibit Ang-1 stimulatory effect on PCNA expression in a dose-dependent manner (Fig. 7B). To confirm that VEGF stimulates an actual increase in cholangiocyte number and to verify if this effect is still evident in ADPKD cholangiocytes, we have studied proliferation in cultured cholangiocytes isolated from the livers of an ADPKD mouse (Fig. 8A) and from an ADPKD patient (Fig. 8B). In ADPKD mice and WT littermates we confirmed the results obtained in rat cholangiocytes. VEGF had a dose-dependent stimulatory effect on cholangiocyte proliferation with a maximum effect at 25ng/mL and Ang-1 administration was synergic to VEGF. In addition, the proliferative effects of VEGF were significantly more pronounced in ADPKD cholangiocytes with respect to WT littermates. The synergic effect of VEGF and Ang-1 was maintained. Similarly, in human cholangiocytes VEGF significantly stimulated proliferation, with a maximum effect at 50ng/mL.

Discussion

The genetic defect causing ADPKD has been identified, but the molecular pathogenesis of liver cyst growth remains unclear. On the basis of the histological features, it has been hypothesized that biliary cyst may derive from embryonic ductal plates that fail to regress properly, and then progressively give rise to cystic lesions in the post-

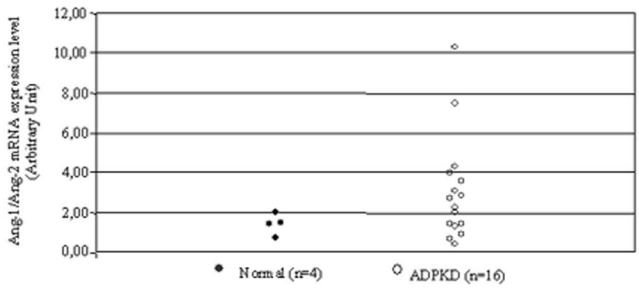

Fig. 6. Expression levels of Ang-1 and Ang-2-related gene transcripts in ADPKD, assessed by real-time PCR and expressed as ratio. The Ang-1/Ang-2 mRNA ratio is increased in ADPKD samples.

natal life.[26] The presence of an aberrant vascularization in close proximity to liver cysts, its association with cyst formation also in the kidney and in the lung,[27,10] where angiogenesis appears to regulate the development of epithelial tissues,[5,10] and preliminary reports showing expression of VEGF by rat cholangiocytes[24] suggested to us that angiogenic factors, *i.e.,* a series of multifunction cytokines capable to regulate the morphogenesis and growth of the vascular system,[7,28] might play a role in cyst development and growth.

On these bases, we have examined the expression of VEGF, angiopoietins and relative receptors in ADPKD and compared it with another morphologically and genetically distinct cystic cholangiopathy and with control livers. Our results show: (1) VEGF and its receptors VEGFR-1 and VEGFR-2, angiopoietin-1 and -2 and their receptor Tie-2 are upregulated in the biliary epithelium of ADPKD; (2) upregulation of VEGF in cystic biliary epithelium is strongly correlated with an increased

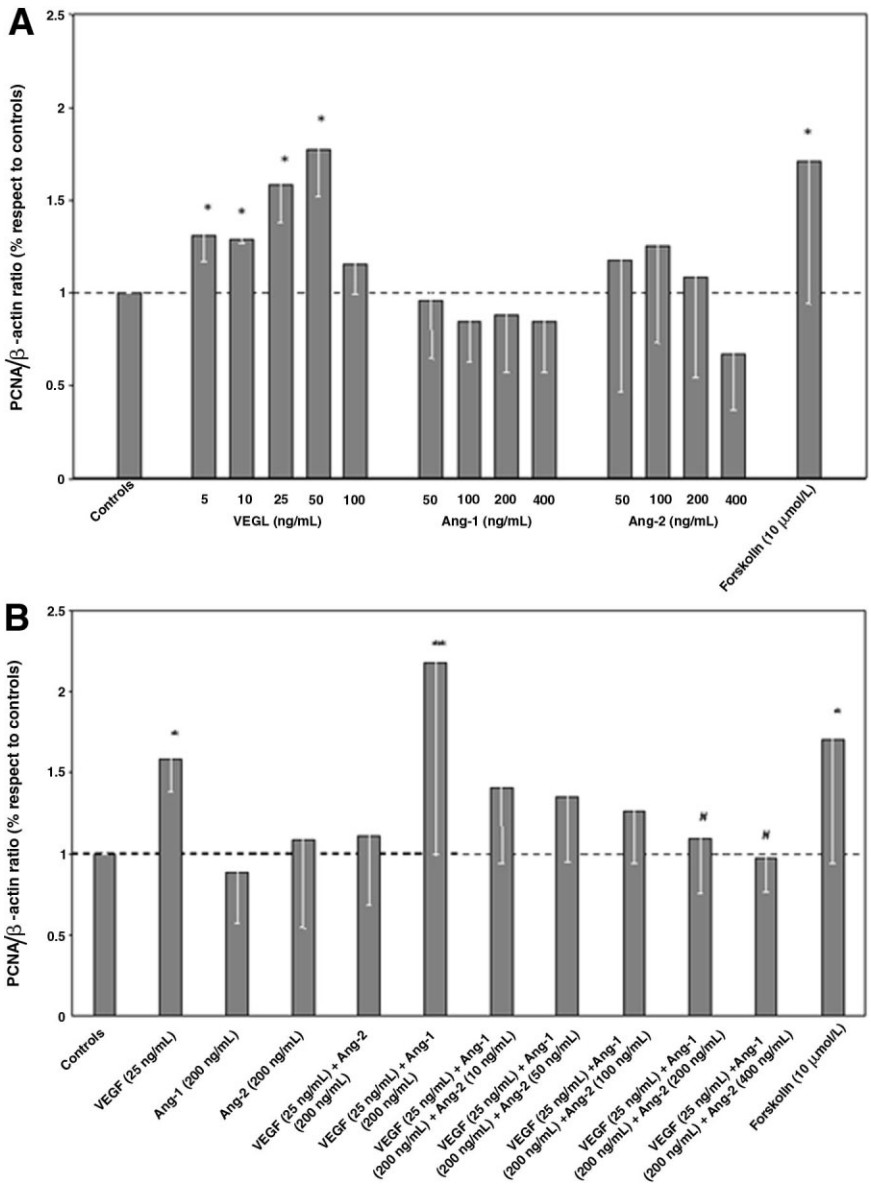

Fig. 7. Proliferative effects measured by PCNA protein immunoblot of VEGF, Ang-1 and Ang-2 on isolated rat cholangiocytes: dose-response curve (A) and effect of their administration as single or mixed solutions (B). VEGF markedly enhances PCNA protein expression in a dose-dependent way and to a similar extent to a known cholangiocyte mitogen (forskolin, 10μmol/L) (A). The strongest proliferative response (nearly 40% greater than forskolin) is obtained with VEGF (25ng/mL) and Ang-1 (200ng/mL) given in combination; notably, the presence of Ang-2 inhibits either VEGF (25ng/mL) or VEGF (25ng/mL) + Ang-1 (200ng/mL) proliferative effects, in a dose-dependent way (B). *$P < .05$; ** $P < .01$; # $P < .05$ vs. VEGF+Ang1 (means ± SD of 4-6 assays per group).

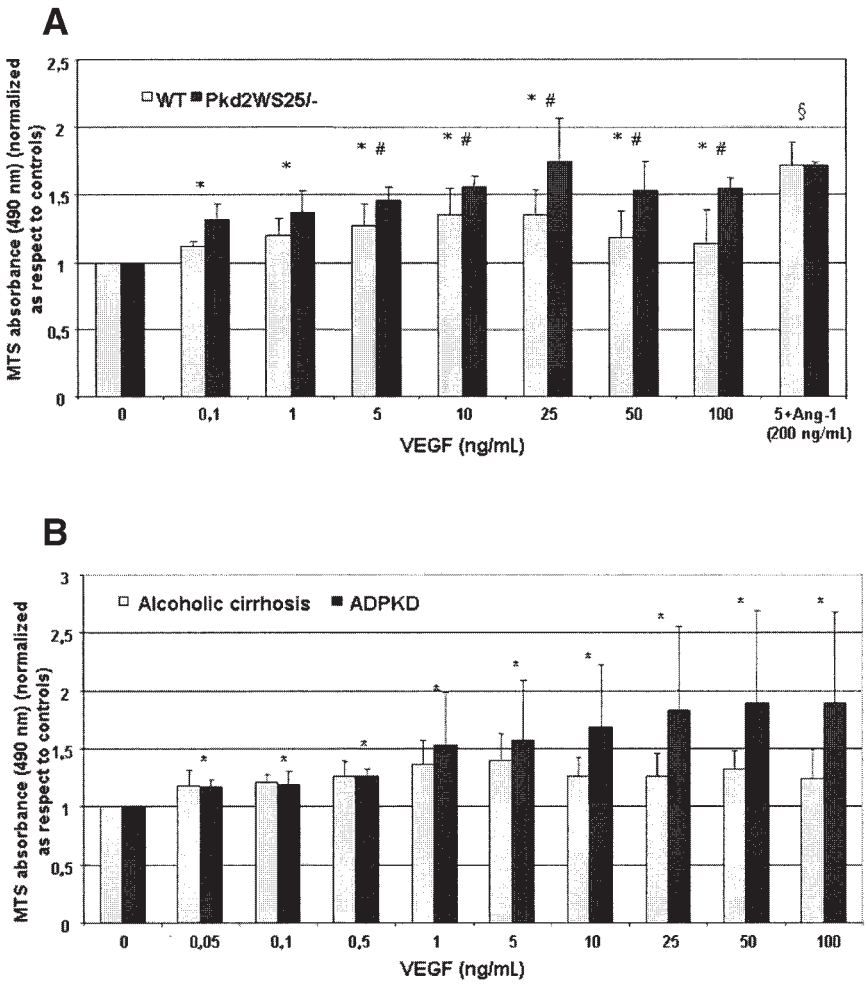

Fig. 8. (A) Proliferative effects, measured by MTS proliferation assay, of VEGF on cultured cholangiocytes isolated from Pkd2$^{WS25/-}$ (black column) and WT mouse (gray column): VEGF significantly enhances the number of cholangiocytes from either ADPKD mouse model or WT with respect to the unstimulated controls (*$P < .05$). Moreover, starting from the concentration of 5ng/mL VEGF significantly stimulates ADPKD cholangiocytes proliferation with respect to the WT (# $P < .05$). Notably, VEGF+Ang-1 showed a significant additive effect with respect to VEGF alone (§$P < .05$) (means ± SD of 4-20 assays per group). (B) Proliferative effects, measured by MTS proliferation assay, of VEGF on cultured human cholangiocytes isolated from ADPKD (black column) and controls (gray column): VEGF significantly enhances the number of cultured living cholangiocytes either from ADPKD or from livers with cirrhosis with respect to the unstimulated cells (*$P < .05$) (means ± SD of 3 to 8 assays per group).

number of portal vascular structures; (3) during embryonic development VEGF, its receptors VEGFR-1 and VEGFR-2, and Tie-2 receptor are expressed by biliary cells in a time-regulated fashion; (4) the Ang-1/Ang-2 gene expression ratio is overall increased in ADPKD; (5) cholangiocytes isolated from ADPKD secrete increased levels of VEGF; and (6) VEGF stimulates cholangiocyte proliferation, an effect strongly enhanced by Ang-1. These data suggest VEGF production by cholangiocytes may promote cyst growth, via an autocrine proliferative effect on the biliary cystic epithelium and via a paracrine stimulation of the vascular supply to the cysts.

Although originally thought to be restricted to vascular cells, we have found that in ADPKD biliary epithelial cells express VEGF, Ang-1, Ang-2 and both types of VEGF receptors along with the angiopoietin receptor Tie-2. We have also shown expression of VEGF and VEGF receptors by the primordial ductal plate cells, consistent with a possible autocrine effect of VEGF on the developing biliary epithelium. While VEGF expression by biliary cells was maintained throughout development, expression of VEGF receptors gradually vanishes during maturation, suggesting that the autocrine effect of VEGF is present only during the early developmental stages of biliary epithelium.

Previous morphological studies underlined the immature aspect of the cystic biliary epithelium. For example, NCAM, an adhesion molecule typically present in immature cholangiocytes[2] is strongly expressed by the cystic epithelium.[2] Expression of VEGF, VEGF receptors as well as Tie-2 on cystic epithelium of ADPKD is yet another feature indicating the lack of maturation of the biliary epithelium in this developmental cholangiopathy. It has been recently demonstrated that polycystin-1 and polycystin-2 are localized in cholangiocyte cilia where they may be involved in the regulation of bile secretion as mechanoreceptors[29] and also influence the transcription of a number of regulatory proteins. We speculate that altered function of polycystins in cholangiocyte cilia may cause a lack of differentiating signals favoring the maintenance of an immature phenotype by biliary epithelial cells. This is consistent with the hypothesis that in the presence of polycystin mutations, the transcription of a number of fetal genes fails to be switched off and thereby

allows the postnatal expression of developmental proteins.[30]

Cholangiocyte co-expression of VEGF, angiopoietins and their cognate receptors, the strong correlation between VEGF expression on abnormal biliary structures and the surrounding vasculature, and the enhanced cholangiocyte capability to secrete VEGF in ADPKD indicates that the aberrant production of angiogenic factors by biliary cysts may, on one side, promote the vascularization of the cysts through a paracrine effect on endothelia, and, on the other side, stimulate the growth of biliary cysts through an autocrine effect on cholangiocytes.

In addition, Ang-1 and, to a lower extent, Ang-2 expression is strongly upregulated in cystic bile ducts of ADPKD. This is a relevant finding because Ang-1, through its interaction with Tie-2, further enhanced the proliferative effects of VEGF on cholangiocytes both in rats and in $Pkd2^{WS25/-}$ mice. The overall effect of angiopoietins on Tie-2 is dependent on a finely tuned local balance between the activating action of Ang-1 and the competitive antagonist action of Ang-2.[31] The critical role played by the balance between Ang-1 and Ang-2 in the liver has been recently addressed in focal nodular hyperplasia,[21] a benign reactive lesion of the liver caused by a vascular malformation.[32] We have quantified the expression level of Ang-1 and Ang-2 transcripts in ADPKD and compared them to control livers. Our results show a specific dysregulation of the angiopoietin balance in ADPKD, with a proportionally higher increase of Ang-1 with respect to Ang-2, resulting in an overall increase of the Ang-1/Ang-2 ratio, consistent with Tie-2 activation in cholangiocytes. Our study also shows that in Caroli's disease immunohistochemical expression of Ang-1 and Tie-2 in biliary epithelial cells is much lower, compared to ADPKD. It is important to underline that from a clinical point of view, in Caroli's disease cysts never reach the huge dimensions typically found in ADPKD.

The biological effects of VEGF and angiopoietins on epithelial cells remain largely to be elucidated. To investigate whether VEGF, Ang-1 and Ang-2 were capable to stimulate biliary epithelial cell proliferation, we have studied the effects on PCNA expression in isolated rat cholangiocytes.[15] In contrast with humans, VEGF receptors and Tie-2 expression is preserved in normal rat cholangiocytes; thus, we could show VEGF caused a clear dose-dependent proliferative response in isolated rat cholangiocytes, an effect that was further increased by co-administration of Ang-1, but was blocked by Ang-2. A similar synergistic effect between VEGF and Ang-1 has been reported on ECs,[33] in which the combined administration of VEGF and Ang-1 resulted in a significant increase of corneal vascularization in comparison with VEGF alone or VEGF plus Ang-2. The proliferative effects of VEGF in cholangiocytes were present also in human cultured cells, isolated from an explanted ADPKD liver and in the cystic epithelium cultured from the $Pkd2^{WS25/-}$ mice. It is therefore our hypothesis that, in ADPKD, VEGF and Ang-1, aberrantly produced by the biliary cells, may promote liver cyst enlargement through an autocrine proliferative effect on cholangiocytes.

In conclusion, taken together these findings indicate that in ADPKD polycystin mutations confer an immature, fetal-like phenotype to the biliary epithelium, characterized by a marked upregulation of VEGF, VEGF receptors, Tie-2 and Ang-1. While on one side angiogenic factors produced by cholangiocytes are responsible for generating the vascular supply to the growing cysts and, as a consequence, for the aberrant vascularization present in polycystic liver diseases, on the other hand they also stimulate the proliferation of the biliary epithelium. This mechanism may be responsible for the progressive biliary cyst enlargement that causes the most severe liver complications of ADPKD. Given the availability of new anti-angiogenic drugs, these results may have important therapeutic implications for ADPKD, suggesting pharmacological interference with VEGF may inhibit disease progression.[10,34]

Acknowledgment: The authors thank Dr. Maria Guido, Prof. Eugenio Gaudio, Prof. Domenico Alvaro and Dr. Giorgio Ballardini for helpful comments and discussion, and Prof. Gianfranco Alpini for his suggestions regarding the PCNA experiments. We are indebted to Dr. Stefan Somlo for the generous gift of $Pkd2^{WS25/-}$ mouse and for helpful discussions.

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