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Stem Cells, Tissue Engineering and Hematopoietic Elements

Analysis of Liver Repair Mechanisms in Alagille Syndrome and Biliary Atresia Reveals a Role for Notch Signaling

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Patients with Alagille syndrome (AGS), a genetic disorder of Notch signaling, suffer from severe ductopenia and cholestasis, but progression to biliary cirrhosis is rare. Instead, in biliary atresia (BA) severe cholestasis is associated with a pronounced "ductular reaction" and rapid progression to biliary cirrhosis. Given the role of Notch in biliary development, we hypothesized that defective Notch signaling would influence the reparative mechanisms in cholestatic cholangiopathies. Thus we compared phenotype and relative abundance of the epithelial components of the hepatic reparative complex in AGS (n = 10) and BA (n = 30) using immunohistochemistry and computer-assisted morphometry. BA was characterized by an increase in reactive ductular and hepatic progenitor cells, whereas in AGS, a striking increase in intermediate hepatobiliary cells contrasted with the near absence of reactive ductular cells and hepatic progenitor cells. Hepatocellular mitoinhibition index ($p21^{waf1}$/Ki67) was similar in AGS and BA. Fibrosis was more severe in BA, where portal septa thickness positively correlated with reactive ductular cells and hepatic progenitor cells. AGS hepatobiliary cells failed to express hepatic nuclear factor (HNF) 1β, a biliary-specific transcription factor. These data indicate that Notch signaling plays a role in liver repair mechanisms in postnatal life: its defect results in absent reactive ductular cells and accumulation of hepatobiliary cells lacking HNF1β, thus being unable to switch to a biliary phenotype. ***(Am J Pathol 2007, 171:641–653; DOI: 10.2353/ajpath.2007.070073)***

Primary cholangiopathies are characterized by chronic ongoing damage to the biliary epithelium. Proliferation of reactive ductules, inflammation, and portal fibrosis coexists with the progressive disappearance of the interlobular/septal bile ducts. Desmet[1] first proposed that this histological lesion, defined as "ductular reaction," was the pacemaker of portal fibrosis and therefore the main mechanism for disease progression in cholangiopathies. Later it was recognized that ductular reactive cells possess distinctive features with respect to "quiescent cholangiocytes" and actively participate to portal inflammation, producing a vast array of cytokines and chemokines, growth factors, and inflammatory mediators that enable them to act as the main nodal point in the cross talk between the different cell components (fibroblasts, endothelial cells, and inflammatory cells) of the "hepatic reparative complex."[2,3]

A human disease that can be considered paradigmatic of this sequence of events is biliary atresia (BA). In this condition, the extrahepatic and major septal bile ducts fail to develop or are destroyed early after birth.[4–8] Despite its heterogeneous etiology, BA represents a stereotypic pathological response, characterized by the generation of a vigorous ductular reaction with development of severe portal fibrosis. The condition rapidly progresses to biliary cirrhosis unless a Kasai operation is performed within the first few months after birth. Often,

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Table 1. Demographic and Clinical Characteristics of Disease Groups

Disease	No. of cases	Age (years)*	Sex (M/F)	Pruritus
AGS	10	1.66† (0.94 to 16.78)	4/6	9/10 (90%)
BA-Tx	17	0.81 (0.50 to 27.70)	9/8	8/17 (47%)
BA-Kasai	13	0.21† (0.08 to 0.94)	4/9	No

ALP, alkaline phosphate; γGT, γ-glutamil transpeptide; OLTX, orthotopic liver transplantation; Tot bil, total bilirubin.
*Median and range.
†$P < 0.05$.
‡$P < 0.01$.
§$P < 0.05$.
¶$P < 0.01$.

(*table continues*)

the Kasai procedure will only reduce the rate of progression, and the patient will receive a liver transplant a few years later.[7,8]

Contrary to BA, patients with Alagille syndrome (AGS) suffer from deep jaundice and severe pruritus as a consequence of the cholestasis caused by congenital intrahepatic ductopenia.[9,10] Progression to liver cirrhosis, however, is slower than in BA, and these patients rarely develop severe manifestations of portal hypertension and transplantation is eventually indicated because of failure to thrive, itching, and hypercholesterolemia.

AGS is caused by mutations in the genes encoding Jagged1, a ligand of the Notch receptors,[11,12] or the Notch-2 receptor itself.[13] There are four Notch receptors, and they can interact with a number of different ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4).[14] These interactions regulate intracellular pathways involved in cell fate decisions[15] during embryonic development of many organs, including the liver. The clinical phenotype in AGS is, in fact, characterized by a wide range of extrahepatic manifestations[9,14,16] in association with severe ductopenia and cholestasis. Experimental studies in mutant mice and zebrafish[17–19] and human genetic studies showed that Notch signaling is required for the biliary tree development during ductal plate remodeling.[17,18,20] Notch signaling seems to control hepatoblasts and mature hepatocytes transdifferentiation into cholangiocytes[21,22] by altering the expression of liver-enriched transcription factors. Interestingly, changes in Jagged1 and Notch expression have been reported also in the course of chronic liver diseases.[23] Mechanisms regulating the regenerative and reparative response to biliary damage determine the long-term outcome of cholangiopathies. Both mature cholangiocytes and hepatocytes are able to proliferate in response to damage, but in most cholangiopathies, ductular reaction dominates the histological picture. Three epithelial phenotypes can be recognized,[24] ie, hepatic progenitor cells (HPCs), intermediate hepatobiliary cells (IHBCs), and reactive ductular cells (RDCs). They can be distinguished by their morphology and pattern of expression of cytokeratin-7 (CK7), a cytoskeletal protein that is absent in mature hepatocytes. HPCs behave as a bipotential transit-amplifying compartment able to differentiate into cells committed toward the hepatocellular (IHBC) or biliary lineage[24,25] (RDC). The cell components of this "hepatic reparative complex" eventually mature into differentiated bile ducts or hepatocytes or may regress by apoptosis if liver damage ceases. In human diseases, all three cellular phenotypes coexist (Figure 1), and their relative enrichment depends on the specific conditions, such as cholangiocyte versus hepatocyte damage and degree of hepatocellular mitoinhibition. These mechanisms, however, have been studied mostly in experimental models of obstructive cholestasis and acute liver injury[3] rather than in human cholangiopathies.

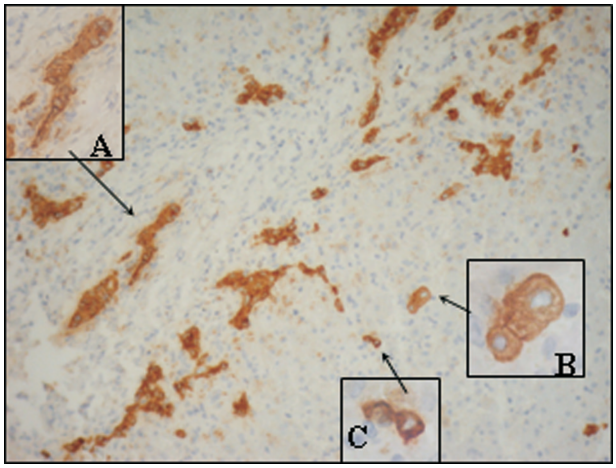

Figure 1. Immunohistochemistry for CK7 in a BA sample, showing the coexistence of the three different epithelial cell types in ductular reaction. **Insets:** RDCs (**A**), IHBCs (**B**), and HPCs (**C**). Original magnification: ×200; **insets:** ×400 (**A**), ×600 (**B** and **C**). Their different morphological properties and classification are detailed in Materials and Methods.

Given the role of Notch signaling in biliary differentiation, we hypothesized that defects in this pathway may generate an imbalance in the cellular elements involved in the regenerative/reparative responses to liver damage. To this aim, using a number of phenotypic markers, we have compared the relative abundance and phenotype of the different epithelial components of the hepatic reparative complex in a congenital deficiency of Notch signaling (AGS) versus a stereotyped response to perinatal biliary damage (BA).

Table 1. *Continued*

Tot bil (mg/dl)*	ALP (<140 U/L)*	γGT (<35 U/L)*	Indication to OLTx
22.35‡ (8.3 to 85.1)	691 (120 to 1092)	434.5§ (37 to 1530)	Growth retardation: 9/10 (90%) Ascites + impaired protein synthesis: 1/10 (10%)
12.6 (0.6 to 44.7)	432 (98 to 750)	151§¶ (17 to 1164)	Growth retardation: 12/17 (70%) Ascites: 6/17 (35%) Impaired protein synthesis: 2/17 (12%) Recurrent cholangitis: 2/17 (12%) Severe portal hypertension: 2/17 (12%)
9‡ (6.9 to 14)	464 (181 to 990)	421¶ (200 to 1127)	

We found important differences between these two cholestatic cholangiopathies. In contrast with BA, AGS was characterized by the absence of ductular reactive cells, by the accumulation of IHBCs that do not express the biliary-specific transcription factor HNF1β and by less advanced fibrosis. These changes are consistent with a profound effect of Notch signaling on liver repair mechanisms during postnatal life. Consistent with the prominent role of the ductular reaction in portal fibrosis, these changes affect the type and extent of liver fibrosis, and the clinical course of the two diseases.

Materials and Methods

Liver Tissue

Frozen samples of AGS (n = 10) and BA-transplanted (BA-Tx) (n = 17) liver tissue were obtained from explants of patients undergoing liver transplantation at the Ospedali Riuniti di Bergamo, Bergamo, Italy. Additional BA samples were obtained at the time of Kasai operation (BA-Kasai, n = 13), at the Ospedali Riuniti di Bergamo. Normal liver (NL) tissue (n = 2) was obtained from potential liver donors, both males aged 49 and 18 years, respectively, whose liver grafts could not be transplanted because of iatrogenic lesions. All diagnoses were based on clinical and laboratory data and on histopathological examination of histological samples.[4,6,16] The demographic, clinical, and biochemical characteristics of patients from the three disease groups are reported in Table 1. Liver tissue was snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C. Informed consent and local ethical committee approval were obtained before tissue collection.

Immunohistochemistry

Phenotypic Markers

Acetone-fixed, 4-μm-thick, serially cut frozen tissue sections were immunostained with antibodies against CK7, CK19, human epithelial antigen-125 (HEA-125), epithelial membrane antigen, and neural cell adhesion molecule; details are given in Table 2. Immunostaining was performed using a two-step procedure with EnVision (DAKO, Milan, Italy).[26] Briefly, after 45 minutes incubation with primary antibodies, sections were sequentially incubated with the proper secondary horseradish peroxidase-labeled antibody (DAKO EnVision) for 30 minutes. DAKO EnVision polymer was used to improve immunoreactivity of the mouse primary antibodies. Immunohistochemical reactions were developed using 0.04 mg/ml 3,3-diaminobenzedine tetrahydrochloride and 0.01% H_2O_2 and counterstained with Gill's Hematoxylin (no. 2; Sigma-Aldrich, St. Louis, MO). In control sections, the primary antibody was omitted.

Proliferation/Mitoinhibition Markers

To study the balance between proliferation and mitoinhibition of hepatocytes, the ratio between Ki67 and p21^{waf1} was determined as described[27] (Table 2). To differentiate hepatocytes from IHBCs, a double-peroxidase immunostaining was performed in acetone-fixed cryosections using CK7. Briefly, after incubation with the primary antibody Ki67 or p21^{waf1}, sections were then incubated with DAKO EnVision for 30 minutes and developed with 3,3-diaminobenzedine tetrahydrochloride, producing a brown staining. Specimens were then sequentially rinsed in distilled water for 15 minutes and 1 mol/L phosphate-buffered saline for 15 minutes, and incubated with anti-CK7 antibody for 45 minutes first and then with DAKO EnVision for 30 minutes; TrueBlue (KPL, Gaithersburg, MD) substrate was used to obtain blue staining of CK7-positive cells.

Hepatocyte and Cholangiocyte Differentiation and Functional Markers

Staining for the hepatocyte nuclear factors HNF4α, HNF6, and HNF1β was performed (see Table 2 for details) to study the biliary and hepatocellular differentiation of the reactive cellular elements in AGS compared with BA. To identify IHBCs and RDCs, double immunostaining for CK7 and HNF1β was performed in selected samples according to the procedure previously described for Ki67 or p21^{waf1}, where CK7 was developed with TrueBlue (KPL) and HNF1β with 3,3-diaminobenzedine tetrahydrochloride. To study coexpression of hepatocyte and cholangiocyte markers in the different reactive cellular elements, dual immunofluorescence staining was per-

Table 2. Primary Antibodies Used for Immunohistochemistry and Biological Significance of the Corresponding Markers

Antibody	Clone	Host	Dilution	Incubation	Supplier	Biological significance
Cytokeratin 7 (CK7)	OVTL-12/30	Ms IgG1	HRP 1:5; IF 1:50	45′	Acris, Hiddenhausen, Germany	Cytoskeletal protein specific of biliary lineage; late expression during biliary ontogenesis
Cytokeratin 19 (CK19)	RCK 108	Ms IgG1	1:20	45′	DAKO	Cytoskeletal protein specific of biliary lineage; early expression during biliary ontogenesis
HEA-125	HEA-125	Ms IgG1	1:100	45′	Progen Biotechnik, Heidelberg, Germany	34-kd epithelial surface glycoprotein (egp34) biliary lineage-specific homologous to nidogen
Epithelial membrane antigen	E29	Ms IgG2a	1:20	45′	DAKO	Group of 250- to 400-kd glycosylated membrane proteins, present in a variety of epithelia of both normal and neoplastic tissues; expressed by mature biliary cells
Neural cell adhesion molecule	UJ13A	Ms IgG2a	1:20	45′	DAKO	Family of cell surface sialo-glycoproteins mediating homophilic and heterophilic interactions in neuroectodermally derived tissues; expressed by immature biliary cells
LKM-1		Hu IgG, FITC-conjugated	1:10	1 hour	Ref. 28	CYP2D6, belongs to hepatocyte-specific cytochrome P450II superfamily; localized in the smooth endoplasmic reticulum
Ki67	Ki-S5	Ms IgG1	1:50	45′	DAKO	Required for maintaining cell proliferation. Localized in the G_1 phase in the perinuclear region, in later phases, it is also detected in the nuclear matrix
$p21^{waf1}$		Ms IgG1	1:50	45′	Oncogene Science, Cambridge, MA	Inhibitor of cellular proliferation in response to DNA damage. It binds and inhibits cyclin-dependent kinase activity, thus preventing their phosphorylation and blocking cell cycle progression
BSEP (ABCB11)		Goat polyclonal	1:100	Overnight	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	Transmembrane protein, selectively expressed by hepatocytes, involved in the ATP-dependent secretion of bile salts into canaliculi
HNF1β		Goat polyclonal	1:300	Overnight	Santa Cruz Biotechnology	Nuclear biliary-specific transcription factor involved in the Jagged1/Notch signal pathway and responsible for the biliary commitment in liver development
HNF4α	K9218	Ms IgG2a	1:100	Overnight	R&D Systems, Minneapolis, MN	Nuclear transcription factor involved in liver and kidney development, selectively expressed by hepatocytes
HNF6		Rabbit polyclonal	1:200	Overnight	Santa Cruz Biotechnology	Nuclear transcription factor expressed by both hepatocytes and cholangiocytes and involved in liver development and morphogenesis

IF, immunofluorescence; Ms, mouse; Hu, human.

formed in selected acetone-fixed cryosections matching anti-CK7 antibody with either anti-LKM-1 fluorescein isothiocyanate (FITC)-conjugated antibody[28] (courtesy of G. Ballardini, Department of Internal Medicine, S. Orsala-Malpighi, Bologna, Italy) or with anti-bile salt export pump (BSEP) antibody[29] (Table 2). Following 1-hour incubation at room temperature with LKM-1 FITC-conjugated antibody or overnight incubation with BSEP, tissue sections were rinsed and then incubated with the secondary antibody FITC-conjugated rabbit anti-goat (dilution, 1:20; incubation, 30 minutes; DAKO). In both cases, tissue sections were then incubated for 45 minutes with the CK7 antibody, detected by the secondary antibody Texas Red-conjugated horse anti-mouse (dilution, 1:20; incubation, 30 minutes; Vector Laboratories, Burlingame, CA). Slides were mounted in glycerol supplemented with 5% 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) to avoid fluorescence bleaching.

Morphometric Analysis

Peroxidase immunostainings were analyzed with a Nikon Eclipse E800 microscope (Nikon, Milan, Italy). Images were collected using a digital camera (Coolpix 995; Nikon), stored by the Fotostation 4.5 software (FotoWare, Oslo, Norway) and analyzed by the Photoshop 5.0 software (Adobe, San Jose, CA) and by the UTHSCSA Image Tool 3.0 (University of Texas, San Antonio, TX). Computer-assisted morphometric analysis was used to quantify RDCs and IHBCs. The different epithelial elements were categorized according to the definition proposed by Roskams et al.[24] RDCs were defined as CK7- and CK19-positive cells with biliary phenotype arranged in irregularly shaped structures.[3,25] IHBCs were defined as cell with morphology and size intermediate between hepatocyte and cholangiocyte, with a peculiar pattern of CK7 immunoreactivity, faint on the cytoplasm and reinforced at the plasma membrane.[24] In digital images of 10 nonoverlapping random fields taken at ×200, the cytokeratin-positive area (CK19 for RDCs and CK7 for IHBCs) was calculated as the percentage of pixels above the threshold value with respect to the total pixels per field; IHBC area was then calculated by subtracting the CK19 to the CK7 area. HPCs were counted as small, oval, or spindle-shaped cells with scant cytoplasm and oval nucleus, alone or in small clamps, localized in the parenchyma or at the portal interface and recognizable by CK19 immunoreactivity.[24,30] The number of HPCs was counted by two independent observers (L.F. and A.S.) in five nonoverlapping random fields observed at ×200.

The proliferative index of hepatocyte and IHBCs was calculated counting the number of nuclei positive for Ki67 in five nonoverlapping random fields taken at ×400. The hepatocyte replicative arrest ratio was expressed according to Clouston[27] as the ratio between the number of $p21^{waf1}$-positive and of Ki67-positive nuclei in five nonoverlapping random fields taken at ×400.

Assessment of Fibrosis

AGS and BA specimens obtained from transplanted patients were assessed with Masson's trichrome staining for the type and extent of fibrosis as detailed below. In each specimen, the septal thickness[31] and the extent of pericellular fibrosis were measured. Septal thickness was measured by computer-assisted analysis (LUCIA G 5.0; Nikon) as the width of the connective tissue scar separating cirrhotic nodules taken in the middle of the septum at ×200 and expressed as micrometers; 10 randomly selected septa were measured for each specimen. The extension of pericellular fibrosis was semiquantitatively scored by two independent observers (M.G. and L.F.) according to the number of positive fields detectable at low magnification (×10) as 0 = absent; 1 = mild (present in one to two fields); 2 = moderate (three to five positive fields); and 3 = severe (more than five positive fields).

Statistical Analysis

Data were expressed as mean ± SD; comparison between groups was calculated using the Student's *t*-test. Correlation was calculated between cell area (RDC and IHBC) and/or number (HPC) and septal thickness (continuous normally distributed variable) using the Pearson's correlation coefficient, whereas nonparametric correlation was calculated between the same cell elements (RDC, IHBC, and HPC) and the extent of pericellular fibrosis (semiquantitative assessment) by the Spearman test. Statistical analysis was performed with SPSS software 13.0 (SPSS Inc., Bologna, Italy), and P values <0.05 were considered as significant.

Results

Alagille syndrome and biliary atresia differ in the extent and quality of cell reaction (see Figure 2). Notable differences were present between AGS and BA. RDCs, significantly increased in BA either at transplant (8.37 ± 6.69%) or before Kasai operation (6.18 ± 4.56%), were nearly absent in AGS (0.45 ± 0.73%) (Figure 2, A–C, G, and I). HPCs, increased in BA (both at transplant and at the time of Kasai operation, 5.45 ± 4.46 and 7.43 ± 5.32, respectively), were rarely observed in AGS (0.64 ± 1.06, $P < 0.0001$). Conversely, IHBCs were remarkably increased in AGS (28.54 ± 17.78%), with respect to BA (both at transplant and at the time of Kasai operation, 10.56 ± 10.55% and 3.01 ± 2.7%, respectively) (Figure 2, D–F and H). Interestingly, a highly significant, direct correlation was found between the number of HPCs and the RDC area ($r = 0.506$, $P < 0.0001$) (Figure 3A). Conversely, an inverse correlation was found between the number of HPCs and the IHBC area ($r = -0.511$, $P < 0.0001$) (Figure 3B). There was no correlation between IHBC area and bilirubin levels ($r = 0.06$, $P =$ not significant), suggesting that differences in IHBC expression were not related to the degree of cholestasis.

Immunophenotype of IHBC was studied by double immunofluorescence using CK7 to identify IHBCs and

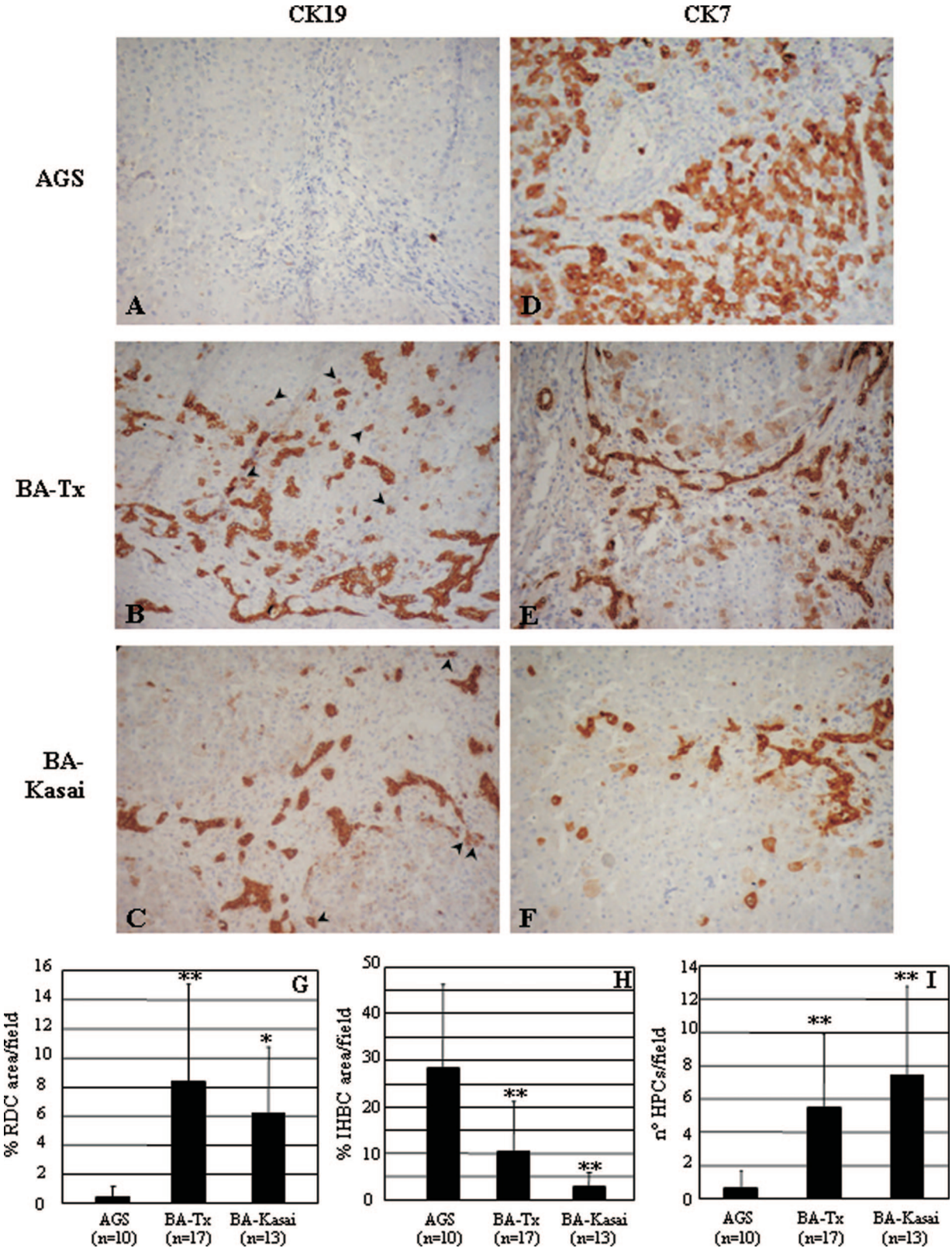

Figure 2. Morphometric quantification of the enrichment in RDCs (**G**), IHBCs (**H**), and HPCs (**I**) in AGS and BA. Representative examples of liver samples immunostained by CK19 (**A–C**) and CK7 (**D–F**) from patients with AGS, BA at transplant, and BA at Kasai are shown. IHBC enrichment in AGS is evident by comparing micrograph **A** (CK19 does not stain IHBCs) with micrograph **B** (CK7 stains all component of the ductular reaction). Micrographs **B** and **E** compare CK19 and CK7 immunostaining in BA taken at the time of transplant; **C** and **F** are the same at the time of the Kasai operation. Note in **E** the periportal localization and lower enrichment of IHBCs. Note also the absence of RDCs and HPCs in AGS (**A**). HPCs are shown as **arrowheads** in **B** and **C**. $^{*}P < 0.01$ versus AGS; $^{**}P < 0.0001$ versus AGS. Magnification, ×200 in all micrographs.

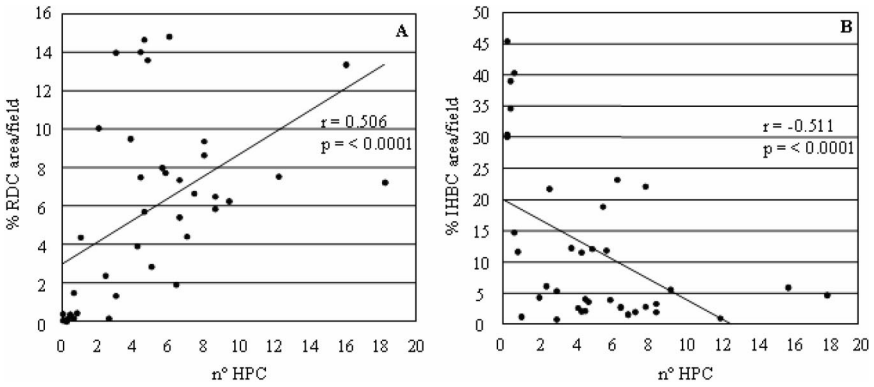

Figure 3. Correlation between the extent of RDCs (**A**) or IHBCs (**B**) and the number of HPCs. The number of HPCs showed a significant positive correlation with the extension of ductular reaction ($r = 0.506$, $P < 0.0001$) (**A**) and a significant but inverse correlation with the expansion of IHBCs ($r = -0.511$, $P < 0.0001$) (**B**). HPCs, RDCs, and IHBCs were measured as outlined in the method section and shown in Figures 1 and 2.

LKM-1 (hepatocyte microsomes) or BSEP (canalicular bile salt export pump) as hepatocellular markers. As shown in Figure 4, in both AGS and BA, IHBCs were positive for LKM-1. BSEP maintained its canalicular polarity, typical of hepatocytes. In BA, IHBCs were mainly localized at the periportal region, where they appeared in strict continuity with CK7-positive/LKM-1-negative ductular reactive cells (RDCs) (Figure 4B). Conversely, in AGS, IHBCs were spread inside the hepatic lobule without any relationship with the rare reactive ductular structures (Figure 4A). Next, we investigated whether the expression of biliary markers in IHBCs from AGS was different from BA. In all groups studied, IHBCs were negative for epithelial membrane antigen and for neural cell adhesion molecule (data not shown).[28] In contrast with AGS, in the BA, part of the IHBCs located at the periportal region were positive for the biliary cell marker HEA-125 (data not shown). As described,[28,32] in most liver diseases, EMA is expressed only by mature cholangiocytes, neural cell adhesion molecules by mature cholangiocytes and RDCs, and HEA-125 by mature cholangiocytes, RDCs, and some IHBCs that are already committed toward a biliary lineage. This is consistent with the lack of expression of HNF1β by IHBCs in AGS but not in BA, in which HEA-125 is detectable. In fact, we also looked at the expression of a number of transcription factors involved in biliary and hepatocellular development. In both cholangiopathies, hepatocytes and IHBCs were positive for HNF4α and HNF6. RDCs and bile ducts were positive for HNF6 and negative for HNF4α (Figure 5). The cholangiocyte-specific transcription factor HNF1β was expressed by RDCs and bile ducts. Interestingly, IHBCs expressed this transcription factor only in BA but not in AGS (Figure 6, A–B). These data support the concept that in AGS, the Notch-dependent block in cell fate determination, whose morphological expression is the accumulation of IHBCs, is localized upstream of HNF1β expression.

Hepatocyte p21^{waf1}/Ki67 Ratio Is Decreased to the Same Extent in Both AGS and BA

It is commonly believed that the progenitor cell compartment is preferentially activated when hepatocyte proliferation is inhibited.[27,33,34] Differences in ductular reaction might therefore be secondary to a different ability of hepatocytes to proliferate. We have therefore studied the ratio between the immunohistochemical expression of p21^{waf1} (an index of decreased cellular replicative capacity) and that of Ki67 (a marker of cell proliferation).[27] Double immunostaining with CK7 and Ki67 or p21^{waf1} was performed to distinguish proliferating hepatocytes from IHBCs. All groups studied were characterized by a significant increase in hepatocyte proliferation (ie, Ki67-positive nuclei) with respect to normal livers (5.64 $\pm$ 2.75 in AGS, 5.58 $\pm$ 3.66 in BA at transplantation, and 12.57 $\pm$ 5.73 in BA at the time of the Kasai procedure, versus 1.60 $\pm$ 0.97 in control livers) (Figure 7A). The hepatocyte replicative arrest ratio (p21^{waf1}-positive hepatocytes/Ki67-positive hepatocytes) did not significantly differ between AGS and BA, both at transplant and Kasai operation (AGS, 0.21 $\pm$ 0.36; BA at transplantation, 0.16 $\pm$ 0.23; BA at Kasai, 0.13 $\pm$ 0.24; NL, 0.62 $\pm$ 0.69; $P < 0.01$) (Figure 7B). This finding suggests that the differences in RDCs and IHBCs between AGS and BA are not related to a different degree of inhibition of hepatocellular proliferation but rather to the intrinsic impaired capability of AGS patient to generate a ductular reaction.

Changes in Pattern of Ductular Reaction Are Associated with Different Patterns and Severity of Liver Fibrosis

Reactive ductular cells are believed to be active participants in the inflammatory and reparative response[3] and in the generation of portal fibrosis during liver damage. Thus, we should expect less fibrosis in AGS where RDCs are nearly absent. To address this question, we have compared septal thickness and the extent of pericellular fibrosis in AGS and BA. We found significant differences between the two conditions: fibrotic septa were much thicker in BA (339.44 $\pm$ 230.39 μm) than in AGS (66.58 $\pm$ 75.42 μm, $P < 0.0001$) (Figure 8, A, B, and E). In contrast, pericellular fibrosis was more extensive in AGS (1.8 $\pm$ 0.79) than in BA (0.35 $\pm$ 0.61, $P < 0.0001$) (Figure 8, C, D, and F). A highly significant, direct correlation was found between septal thickness and RDC area ($r = 0.742$, $P < 0.0001$) (Figure 8G) and HPC number ($r = 0.605$, $P < 0.0001$), whereas the IHBC area indirectly correlated with septal thickness ($r = -0.620$, $P < 0.0001$)

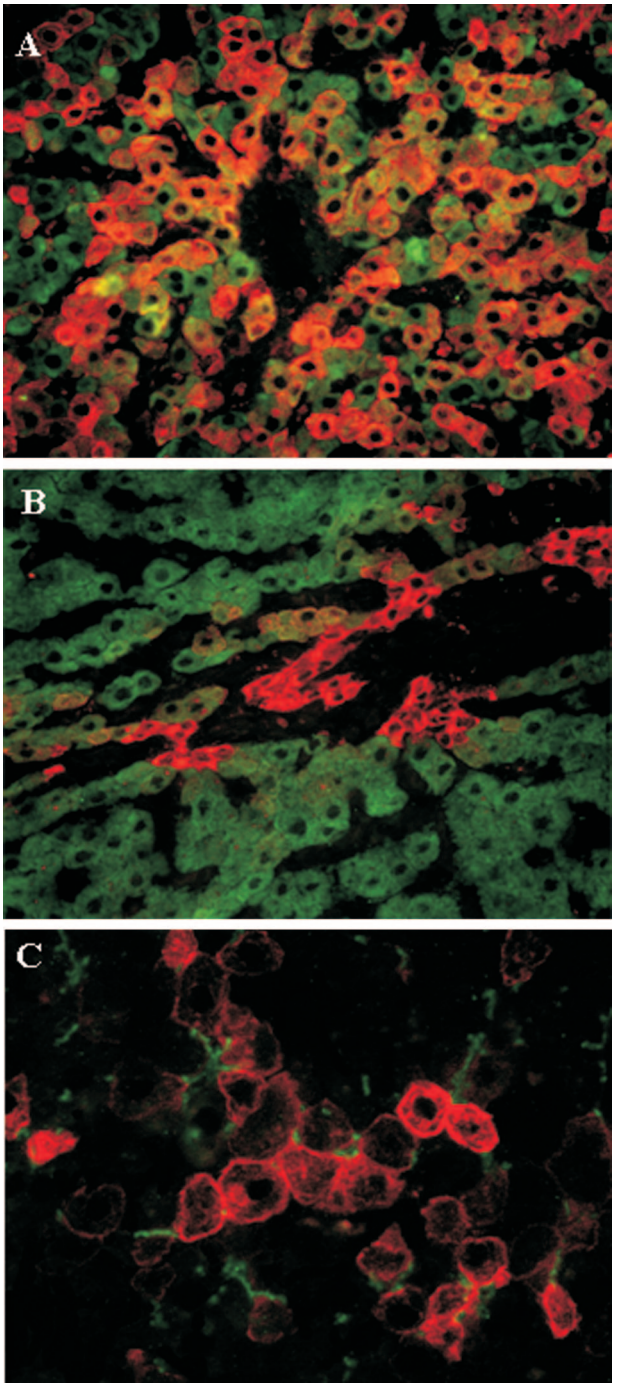

Figure 4. Dual immunofluorescence staining for CK7 (tetramethylrhodamine B isothiocyanate) and the hepatocellular microsomal marker LKM-1 (FITC) in AGS (**A**) and BA (**B**). In **C**, dual immunofluorescence staining for CK7 (tetramethylrhodamine B isothiocyanate) and the hepatocellular canalicular marker BSEP (FITC) in AGS is shown. IHBCs clearly express hepatocellular markers (LKM-1, cytoplasmic; and BSEP, canalicular) and are much more represented in AGS than in BA. In BA, they are mainly located in the periportal area, in strict contiguity with CK7-positive/LKM-negative/BSEP-negative ductular cells. Magnification: ×400 (**A** and **B**), ×600 (**C** and **D**).

(Figure 8H). Pericellular fibrosis showed a significant negative correlation with both RDCs ($r_s = -0.519$, $P < 0.006$) and HPCs ($r_s = -0.436$, $P < 0.05$). These results suggest that the presence and extent of ductular reaction is a major determinant of portal fibrosis in cholestatic cholangiopathies.

Discussion

In chronic liver disease there is a continuous interaction between ongoing liver cell damage and regenerative/reparative mechanisms. Understanding the mechanisms of liver repair and regeneration in chronic liver disease is a fundamental step in the attempt to preserve organ function and to prolong survival of liver patients.[3] The liver is a peculiar organ, because its normal tissue maintenance is driven by division of mature epithelial cells (hepatocytes or cholangiocytes). However, most forms of chronic liver diseases show various degrees of "ductular reaction," a sign of liver progenitor cell activation. A prominent aspect of this histological lesion is the presence of a range of epithelial cells (with phenotypes between immature cholangiocytes and hepatocytes) that maintain intimate anatomical contacts with mesenchymal, inflammatory, and endothelial cells.[3] These epithelial cells are believed to represent a transit compartment generated by the expansion of hepatic progenitor cells.[3] Early experiments in rodents led to the belief that a progenitor (or "oval") cell reaction was activated only in case of massive hepatocyte loss or of important inhibition in hepatocyte proliferative ability.[25] Studies in humans, however, have shown that progenitor cells are activated in the majority of liver diseases, even in the presence of minimal degree of liver damage.[27,35,36] The mechanisms promoting this reaction and the histogenesis of its epithelial cell components are still largely unknown. Hepatic progenitor cells are believed to be bipotential and able to differentiate along the hepatocytic or biliary lineage, via intermediate hepatobiliary cells or via the formation of atypical reactive ductules, respectively.[30] However, there is morphological evidence suggesting that reactive ductules can also be generated from biliary transdifferentiation of hepatocytes (so-called ductular metaplasia).[21,22,37–39] In this study, we investigated the phenotypic differences in the epithelial components of the hepatic reparative complex between two developmental cholestatic cholangiopathies, AGS and BA, occurring at similar ages, but with important differences in their clinical course and progression to biliary cirrhosis.

AGS is caused by mutations of a Notch ligand, Jagged1, or of Notch-2 itself. Notch proteins are a group of transmembrane receptors involved in liver development. Given the role of Notch signaling in cell fate determination and in bile duct development,[15,17,18,20] we hypothesized that defective Notch signaling would have an impact on the liver reparative complex. In fact, by comparing the different cell populations participating in liver repair mechanisms in AGS and BA, we observed that AGS was characterized by a marked expansion of IHBCs and by the near absence of RDCs and HPCs, which were significantly increased in BA.

The dramatic increase in IHBCs represents the most prominent change we observed in AGS. These changes are not secondary to different degrees of cholestasis,

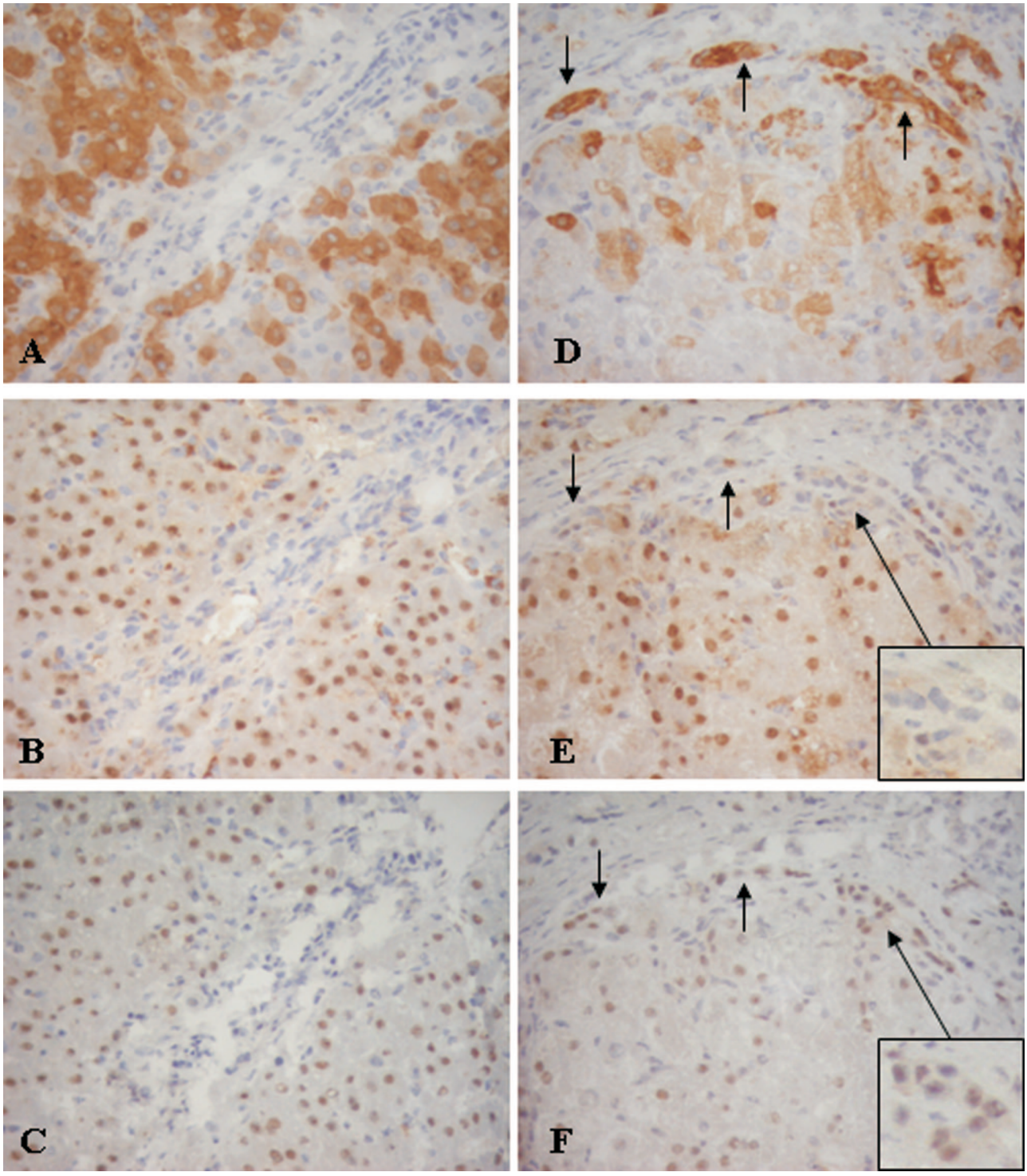

Figure 5. Cell expression of HNF4α and HNF6 in serial sections from AGS and BA. RDCs, HPCs, and IHBCs are identified by immunoreactivity for CK7 (**A** and **D**). In both cholangiopathies (AGS: **A–C**, and BA: **D** and **E**), HNF4α (**B** and **E**), and HNF6 (**C** and **F**) share a similar pattern of expression, where hepatocytes and IHBC are positive for HNF4α and HNF6, whereas RDCs (**arrows**, see also insets in **E** and **F**) and bile ducts are positive for HNF6 and negative for HNF4α. Magnification: ×400 in all micrographs, ×600 in **insets**.

because bilirubin levels were similar in AGS and BA, and there was no correlation between serum bilirubin concentration and IHBC area. These changes were also not related to different levels of hepatocellular mitoinhibition, as judged from the $p21^{waf1}$/Ki67 index. We actually found that hepatocyte proliferation was increased to a similar extent in both AGS and BA, with respect to controls. Thus, the abundance of IHBCs in AGS is likely a direct consequence of the defective Notch signaling. Among the four Notch receptors, Notch-2 is more likely involved in the pathogenesis of AGS. In fact, Notch-2 mutations have been described in families with AGS phenotype and

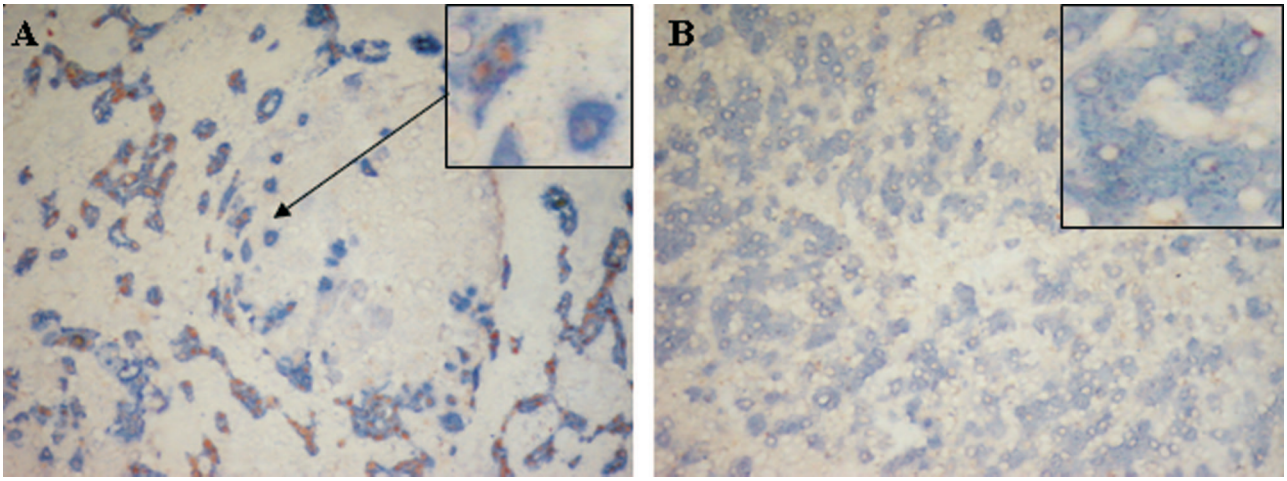

Figure 6. Immunophenotype of IHBC is different in AGS compared with BA. **A** shows the expression of the HNF1β transcription factor (a transcription factor regulated by Jagged1/Notch signaling and involved in biliary differentiation) by IHBCs, RDCs, and HPCs in a representative case of biliary atresia. **B** shows instead the lack of expression of the HNF1β transcription factor by IHBCs in a representative sample of AGS. Double immunostaining for CK7 (TrueBlue) and HNF1β (horseradish peroxidase). Magnification: ×200 in all micrographs, ×600 in **insets**.

no Jagged-1 mutations.[13] Furthermore, mice with heterozygous Jagged-1 and hypomorphic Notch-2 alleles present a phenotype orthologous to AGS.[40] In addition, during biliary development Jagged-1 is expressed on cells in portal mesenchyme and signals to the Notch-2 receptor expressed in hepatoblasts.[21] Notch-2 signaling then orchestrates the activation of genes favoring biliary development, including HNF1β, and the repression of genes favoring hepatocellular differentiation.[41,42] Therefore, our data support the hypothesis that, in addition to its known involvement in liver ontogenesis, Notch plays a critical role also in hepatic repair mechanisms during postnatal life.

Our data also shed some light on the histogenesis of the hepatic reparative complex. The accumulation of IHBCs in the near absence of RDCs in AGS could indicate that deficient Notch-2 signaling affects the ability of HPCs to generate RDCs. However, if this were the case, HPCs, rather than IHBCs, would increase in number and accumulate. Alternatively, if the role of Notch pathway was to "stabilize" stem cells, preventing their differentiation,[43] IHBCs would be generated by the unrestricted activation of liver stem cells unable to differentiate along the cholangiocyte lineage, but it would be unclear why they would accumulate rather than progress in their hepatocellular differentiation.

Instead, the high proliferative activity of hepatocytes, the lack of HNF1β expression in IHBC from AGS, and the negative correlation between HPCs and IHBCs suggest that Notch signaling may be required for the transdifferentiation of hepatocytes into RDCs.[21,22,37–39] During development, Notch signaling has an inductive effect on hepatoblasts, promoting their differentiation into biliary epithelial cells.[21] In addition, data in organoid cultures[37,38] and chimeric rodent models[39] demonstrate that mature hepatocytes can transdifferentiate into biliary cells. The strong excess of cells adopting an intermediate phenotype in AGS was likely derived from proliferating hepatocytes, unable to form ductular structures due to a lack of Notch signaling. Thus, in a sort of recapitulation of ontogenesis, proliferating hepatocytes, rather than HPCs, may be the source of IHBCs in AGS.[28,44,45] These "stem cell properties" of mature hepatocytes are well known,[28,46] and there is now increasing evidence that hepatocytes are actually capable to transdifferentiate into biliary epithelial cells *in vitro*[37,38] and *in vivo* in conditions of experimental severe bile duct damage.[39] Nishikawa et al[22] in mature hepatocytes and Tanimizu et al[21] in hepatoblasts have shown that this process is accompanied by the activation of the Notch signaling pathway, up-regulation of HNF1β, and down-regulation of HNF1α and

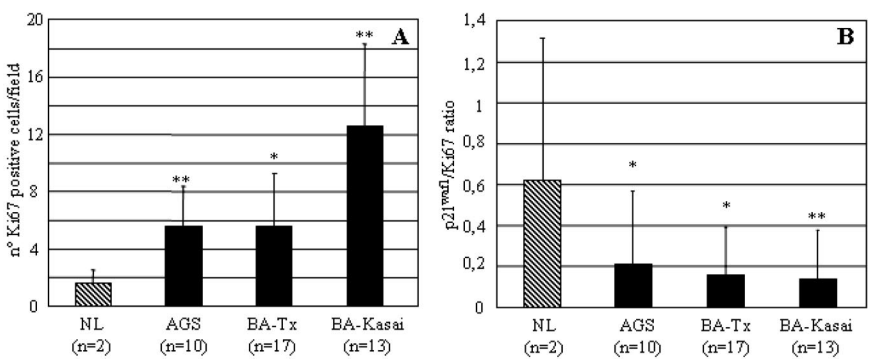

Figure 7. Proliferation (Ki67-positive nuclei) and replicative arrest ratio (p21/Ki67-positive nuclei) of hepatocytes in AGS, BA-Tx, and BA-Kasai compared with NL. In all diseases, hepatocyte proliferation (**A**) was markedly enhanced with respect to NL (dotted column); moreover, the hepatocyte replicative arrest ratio was significantly reduced in AGS, BA-Tx, and BA-Kasai with respect to NL (**B**). *$P < 0.01$ versus NL; **$P < 0.0001$ versus NL.

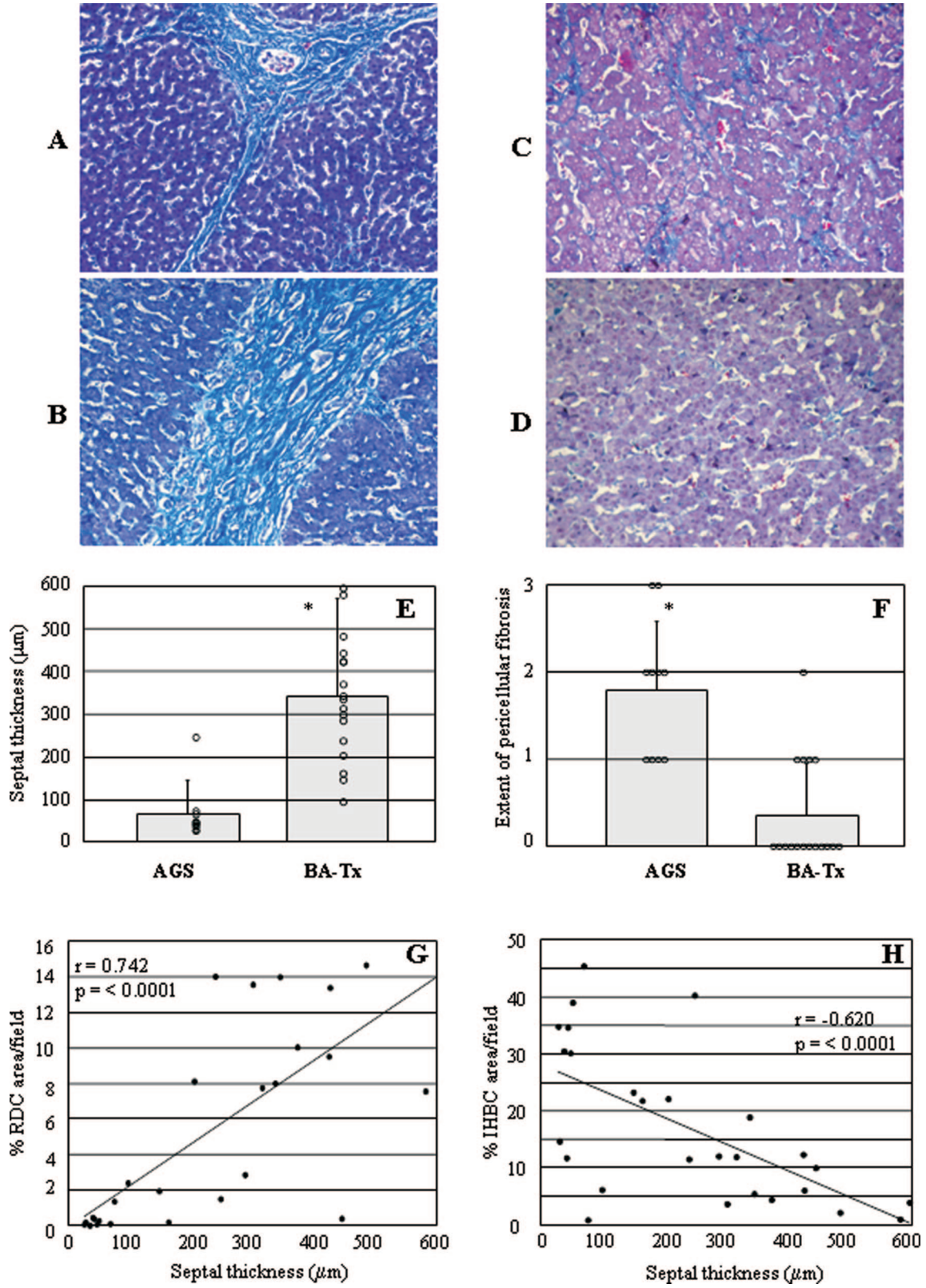

Figure 8. Different pattern of fibrosis between AGS (**A** and **C**) and BA (**B** and **D**) (Masson's trichrome staining; magnification, ×200) expressed by septal thickness (**A** and **B**) and pericellular fibrosis (**C** and **D**). The column plots below show the mean (±SD) and data distribution for septal thickness (in micrometers) (**E**) and pericellular fibrosis (semiquantitative assessment, see Assessment of Fibrosis under Materials and Methods for details) (**F**) in AGS and BA. Statistical significance (*$P < 0.01$) calculated by using the Student's *t*-test. Plots in **G** and **H** show the correlation between the septal thickness and the extent of RDCs (**G**) and IHBCs (**H**). Septal thickness significantly and directly correlated with the extent of RDCs ($r = 0.742$, $P < 0.0001$) and inversely with the IHBC area ($r = -0.620$, $P < 0.0001$) (**A** and **B**).

HNF4. These are transcription factors involved in hepatocyte and cholangiocyte differentiation.[41] In particular, HNF4 and HNF1α are necessary for hepatocellular differentiation, whereas HNF1β is involved in cholangiocyte differentiation. Our results are consistent with data showing that HNF1β expression is regulated by Notch-2.[21,41]

Our study also provides additional evidence for a major role of the hepatic reparative complex in portal fibrosis associated with chronic cholestatic cholangiopathies. In the last decade, it has become clear that reactive ductular cells are active participants to the inflammatory response to liver damage by producing a vast array of proinflammatory and fibrogenetic chemokines and growth factors.[2,3,47] These mediators enable the extensive cross talk between epithelial, mesenchymal, and endothelial cells required to generate the reparative response.[3] Recent studies have shown that, in addition to acute fulminant hepatitis and chronic cholangiopathies, HPCs and RDCs are also activated in chronic hepatitis of diverse etiologies (autoimmune, alcoholic and nonalcoholic steatohepatitis, hepatitis C virus, and hepatitis B virus).[33,48] These studies have shown that the extent of ductular reaction correlates with the histological staging of fibrosis, but a cause-effect relationship has not yet been demonstrated. In this study, we have analyzed the relationship between the epithelial components of the reparative complex and portal fibrosis in two different cholestatic cholangiopathies. We show that RDCs and HPCs correlate with septal thickness but not with pericellular fibrosis. Thick septa are characteristic of BA where cholestasis coexists with a strong expansion of RDCs, whereas thin septa with prominent pericellular fibrosis are a feature of AGS where cholestasis is not accompanied by an increase in RDCs. This observation has important clinical implications and may explain the different clinical course of these two cholestatic conditions. In fact, septal thickness has been shown to correlate with the severity of cirrhosis and the presence of clinically relevant portal hypertension.[31] The observation that portal fibrosis was drastically reduced in the absence of a ductular reaction is consistent with the intimate relationships between reactive ductules and mesenchymal and inflammatory cells and their extensive cross talk.

The clinical picture and natural history of Alagille syndrome are quite heterogeneous.[9,14,16] Clearly, our study is based on the 30% of AGS patients that require liver transplantation and therefore may have more progressive liver disease.[49] However, in AGS the indication for liver transplantation is most often for failure to thrive, itching, and severe hypercholesterolemia rather than for liver cirrhosis and its complications. Although only 30% of the cases require liver transplantation, cholestasis and ductopenia are present in approximately 90% of the patients.[9,10] Our working model would predict that cases with less progressive liver disease are affected by a more profound Notch signaling defect and have an even more reduced ductular reaction.

A number of aspects in AGS pathogenesis are still unresolved. In particular, it is unclear whether ductopenia is caused by the developmental lack of bile ducts or by the progressive loss of previously normal bile ducts. Intrahepatic bile ducts appear to develop normally during the fetal period in AGS, and analysis of cases with sequential biopsy specimens suggests that paucity develops with increasing age.[10,50] Libbrecht et al[51] described a case with a clear gradient in the level of ductopenia. Ductopenia was more severe at the periphery of the liver, whereas normal ducts were seen in the central, hilar region. These authors also described hypertrophic arteries in the portal spaces with ductopenia and normal arteries in the hilar region.[51] Although randomly taken, our liver samples were characterized by arterial hypertrophy, suggesting that they were sampled from the periphery.

Libbrecht et al[51] hypothesized that AGS is characterized by a lack of postnatal development of the terminal branches of the bile ducts. We suggest that ductopenia may also result from the inability of patients with AGS to mount a ductular reaction and repair biliary damage. In AGS, a defective Jagged1/Notch-2 signaling leads to an accumulation of intermediate hepatobiliary cells unable to transdifferentiate into biliary cells. This type of response yields a peculiar clinical significance, since it proceeds with a form of reparative/fibrotic reaction characterized by thin septa and pericellular distribution. The differences in regenerative/reparative mechanisms may explain the different clinical characteristics of AGS and BA.

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