Histol Histopathol (2015) 30: 205-212
DOI: 10.14670/HH-30.205

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Angiogenesis: a new surrogate histopathological marker is capable of differentiating between mild and significant portal hypertension

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Summary. Aim: Angiogenesis is considered an important pathophysiological feature of portal hypertension. We investigated the ability of angiogenesis, as CD34-positive microvessel density (MVD), to differentiate portal pressure in a CCl_4-induced rat cirrhosis model. Methods: Cirrhosis was induced by intraperitoneal injection of carbon tetrachloride in 46 male adult Sprague-Dawley rats. A catheter connected to a highly sensitive pressure transducer was inserted into the portal vein to continuously record portal pressure. Fibrosis area, nodule size and MVD were assessed by image morphometry. Results: Of 42 rats in which portal pressure was measured successfully, 27 (64%) had portal pressure ≥10 mmHg, defined as significant portal hypertension. MVD was 4.5-fold higher and fibrosis area 13.0-fold higher in rats with significant portal hypertension than in rats with portal pressure <10 mmHg. Portal pressure was significantly correlated with MVD ($r=0.491$, $p<0.001$) and fibrosis area ($r=0.545$, $p<0.001$) in all animals, but only MVD correlated with portal pressure ($r=0.731$ $p<0.001$) in rats with significant portal hypertension. The area under receiver operating characteristic curve for MVD in all rats was 0.953 (95% CI: 0.875-1.031) and optimum cutoff for MVD was 18/mm^2, with 96.3% sensitivity and 93.3% specificity. Conclusions: We found that MVD, measured by CD34 immunostaining, was better able than the fibrosis area to discriminate significant portal hypertension in rats, suggesting that MVD could be a surrogate marker for portal hypertension in patients with liver diseases.

Key words: Cirrhosis, Fibrosis area, Microvessel density, Nodule size, Portal hypertension

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Introduction

Portal hypertension, resulting in esophageal varices and ascites, is one of the most important complications of cirrhosis. Most deaths in cirrhotic patients are due to portal hypertension (Cardenas and Gines, 2009; Thabut and Shah, 2010). Moreover, portal pressure is a determinant factor in the natural history of cirrhosis and predicts patient prognosis (Snowdon et al., 2012; Rastogi et al., 2013). To date, measuring hepatic venous pressure gradient (HVPG) has been considered the most adopted method of assessing portal pressure (Ripoll et al., 2007). This procedure, however, is invasive, uses specialized technology and requires a skilled interventional radiologist. It is therefore rarely performed, especially in developing countries, which have high endemic rates of liver diseases (Groszmann and Wongcharatrawee, 2004; Thalheimer et al., 2011). Identification of surrogate markers of HVPG that can discriminate portal pressure is therefore imperative. These markers may allow clinicians to more easily evaluate the severity of cirrhosis and select appropriate treatment options for patients with liver diseases.

Various clinical (de Bruyn and Graviss, 2001), serological (Mal et al., 1998) and imaging (Carrion et

al., 2006; Nedredal et al., 2011) parameters have been assessed for their relationship to HVPG. Recently, however, efforts have been made to show relationships between HVPG and histopathological parameters of liver biopsies from cirrhotic patients (Nagula et al., 2006; Kumar et al., 2008; Calvaruso et al., 2009; Sethasine et al., 2012; Rastogi et al., 2013). These studies have shown that fibrosis area, small nodularity and septal thickness correlate with HVPG. Furthermore, fibrosis area and nodular size were found to distinguish clinically significant portal hypertension, defined as HVPG ≥10 mmHg, consistent with clinical evidence of portal hypertension such as the appearance of esophageal varices.

Hepatic microvessel changes rather than fibrosis itself are regarded as important pathophysiological features of portal hypertension (Fernandez et al., 2009; Valfrè et al., 2009). Angiogenesis, a key change in vasculature during cirrhosis, has been shown to contribute to the development of portal hypertension (Thabut and Shah, 2010). Moreover, the concentrations of angiogenic factors, including vascular endothelial growth factor and platelet derived growth factor, were found to increase in proportion to the severity of liver diseases (Jaroszewicz et al., 2008; Diang et al., 2012). We therefore hypothesized that quantitative analysis of angiogenesis, as shown by CD34 immunostaining, could more accurately evaluate portal pressure than fibrosis area and nodule size. Using a rat model of carbon tetrachloride (CCl4) induced cirrhosis, we therefore assessed the correlation between the degree of angiogenesis, i.e. CD34-positive microvessel density (MVD) and portal pressure, to determine if MVD could discriminate portal hypertension in these animals.

Materials and methods

Induction of cirrhosis by CCl_4

Forty-six male adult Sprague-Dawley rats, weighing 180-220 g, were maintained in an environmentally controlled room (23±2°C, 55±10% humidity) with a 12-hour light/dark cycle and free access to food and water. Cirrhosis was induced by intraperitoneal (i.p.) injection of 2 ml of a mixture of CCl_4 and olive oil (4:6, v/v) per kg body weight, which results in a high degree of micro nodular cirrhosis after approximately 12 weeks. After administration of CCl_4 for eight weeks, ascites and mesenteric collateral circulation developed in a majority of rats once portal pressure was over 10 mmHg. We therefore used 10 mmHg as a cut-off value to distinguish rats with mild or significant portal hypertension. The rats were euthanized at 0 (control), 4, 8, and 12 weeks. All animal experiments were performed in accordance with the guiding principles for the care and use of laboratory animals approved by the Research Ethics Committee of the Beijing Friendship Hospital, Capital Medical University, China (Permit Number: 12-1004).

In vivo hemodynamic measurement of portal pressure

A catheter (ID 0.86 mm, OD 1.27 mm) was inserted into the portal vein of each rat via the ileocolic vein (Castañeda et al., 2000; García-Calderó et al., 2011). The catheter was connected to a highly sensitive pressure transducer (BL-420S Physiological Systems, Taimeng Instruments, Chengdu, China), which was used to continuously record portal pressure during a 10-minute stabilization period (Zhang et al., 2012). The animals were euthanized as above with an overdose of sodium pentobarbital and their livers and spleens were removed and weighed. All livers were subsequently assessed histopathologically.

MVD by CD 34 immunostaining

Sections were incubated with goat polyclonal antibodies against rat CD34 (LS-C150289, LifeSpan BioSciences, Seattle, WA, USA) and expressed as the mean number of CD34-positive microvessels per mm^2 (Fig. 1A). Weidner et al. reported a significant direct association between the incidence of metastasis in patients with breast cancer and microvessel density (MVD) (Weidner et al., 1991). This study measured MVD by light microscopy at under 200× magnification in a single tumor invasive area, with positive microvessels counted and called MVD. In our study, MVD was calculated by a modification of Weidner's method, as described below. The areas containing the highest numbers of stained positive microvessels, which were treated as 'hot spots', were first identified at low magnification (×10). Once five 'hot spots' were recognized, individual positive microvessels were automatically counted under magnification (×20) with the aid of IPP software. Any brown-staining endothelial cells or endothelial cell clusters, clearly separate from adjacent microvessels and other connective-tissue elements, were considered single countable microvessels (Maeda et al., 1996). In our study, vessel lumens were not necessarily defined as individual microvessels, and red cells were not used to define vessel lumens. Our MVD results were therefore the average number of positive micro vessels calculated from five 'hot spots'.

Histopathological assessment of fibrosis and nodule size

Liver samples were routinely fixed in formalin and embedded in paraffin, and the slides were stained for collagen with Sirius-Red. Images were captured and evaluated at 4× magnification for fibrosis area and nodule size by a single investigator blinded to the results of portal pressure measurement (Sethasine et al., 2012). Fibrosis area was calculated as the ratio of the area of fibrosis to the total sample area and expressed as a percentage (Fig. 1B), whereas nodule size was expressed as the sum of the mean maximum lengths (in mm) of all nodules in that sample, after excluding incomplete

nodules (Fig. 1C).

Experimental settings for image morphometry

Fibrosis area, MVD and nodule size were quantitatively assessed with a microscope (Olympus BX53; Olympus, Tokyo, Japan) and a digital camera recorder (Olympus DP72) connected to a computer. All acquired images were analyzed using Image Pro-plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

All statistical analyses were performed using SPSS 16.0 statistical package (IBM, Chicago, IL, USA), with a p value <0.05 considered statistically significant. Continuous variables were expressed as median (P_{25}-P_{75}) and compared in rats with portal pressures <10 mmHg and ≥10 mmHg using the Mann-Whitney U test. Correlations between variables were evaluated using Pearson's or Spearman's coefficient analysis. Variables with p<0.05 were entered into a stepwise multivariable regression analysis. Receiver operating characteristic (ROC) curve analysis was used to maximize the sensitivity and specificity of MVD in discriminating between mild and significant portal hypertension, defined as portal pressure ≥10 mmHg (Youden, 1950; Hilden and Glasziou, 1996).

Results

A comparison of rats with portal pressure <10 mmHg and ≥10 mmHg

We successfully measured portal pressure in 42 of the 46 rats treated with CCl_4. Mean portal pressure from normal control rats was 6.3mmHg (n=8) and median value of portal pressure was 11.52 mmHg in all rats (n=42). Twenty-seven (64%) had portal pressure ≥10 mmHg, which is defined as significant portal hypertension, with the other 15 (36%) having portal pressure <10 mmHg. MVD was 4.5-fold higher, fibrosis area 13.0-fold higher, liver weight 1.3-fold higher and spleen weight 2.2-fold higher in the rats with portal pressure ≥10 mmHg than in those with portal pressure <10 mmHg (Table 1). Body weight, however, was similar in these two groups.

Relationship between portal pressure and histopathological parameters in all rats

Univariate analysis identified three parameters that significantly correlated with portal pressure: MVD (r=0.867, p<0.001), fibrosis area (r=0.834, p<0.001) and spleen weight (r=0.656, p<0.001). Portal pressure was also weakly correlated with liver weight (r=0.349, p=0.023), but did not correlate with nodule size(r=-0.371, p=0.052) (Table 2). On multivariate analysis, only MVD (r=0.491, p=0.002) and fibrosis area (r=0.545, p<0.001) were independently predictive of portal pressure. Stepwise multiple regression analysis showed that MVD (r=0.408, p=0.002) and fibrosis area (r=0.441, p<0.001) were independently correlated with portal pressure.

Relationship between portal pressure and histological parameters in rats with significant portal hypertension

Univariate analysis of the 27 rats with significant portal hypertension showed that only MVD significantly correlated with portal pressure (r=0.778, p<0.001). A weak correlation was observed between portal pressure and fibrosis area (r=0.429, p=0.026), but there was no correlation between portal pressure and either liver (r=-0.161, p=0.422) or spleen (r=0.177, p=0.378) weight (Table 2). The correlation between portal pressure and nodular size was also analyzed in 26 rats with significant portal hypertension, but no correlation was observed (r=-0.264, p=0.193). On multivariate analysis, only MVD (r=0.731, p<0.001) were independently predictive of portal pressure. Multivariate regression analysis showed that only MVD was independently correlated with portal

Table 1. Histological parameters, body weight, liver and spleen weight and portal pressure in rats with portal pressure <10 mmHg and ≥10 mmHg.

Parameters	Portal Pressure <10 mmHg (n=15)	Portal Pressure ≥10 mmHg (n=27)	p value
Microvessel density (number/mm^2)	13.16 (9.14-19.56)	56.47 (30.81-66.92)	<0.001
Fibrosis area (%)	0.94 (0.39-2.66)	12.25 (8.45-15.50)	0.001
Body weight (g)	404.6 (299.9-463.9)	428.5 (388.2-480.8)	0.138
Liver weight (g)	10.98 (9.89-13.13)	14.30 (12.12-15.87)	0.001
Spleen weight (g)	0.93 (0.89-1.04)	2.03 (1.64-2.56)	<0.001

Results are expressed as median (P_{25}-P_{75}).

Table 2. Correlations between histological parameters and liver and spleen weight with portal pressure in rats with portal pressure<10 mmHg and ≥10 mmHg.

Parameters	All Rats		Portal Pressure ≥10 mmHg	
	Pearson's correlation coefficient (n = 42)	P value	Pearson's correlation coefficient (n=27)	P value
Microvascular density (number/mm^2)	0.867	<0.001	0.778	<0.001
Fibrosis area (%)	0.832	<0.001	0.429	0.026
Nodule size (mm)	–0.371	0.052	–0.264	0.193
Liver weight (g)	0.349	0.023	–0.161	0.422
Spleen weight (g)	0.656	<0.001	0.177	0.378

Nodules not fully formed in rats with mild or moderate portal hypertension were excluded.

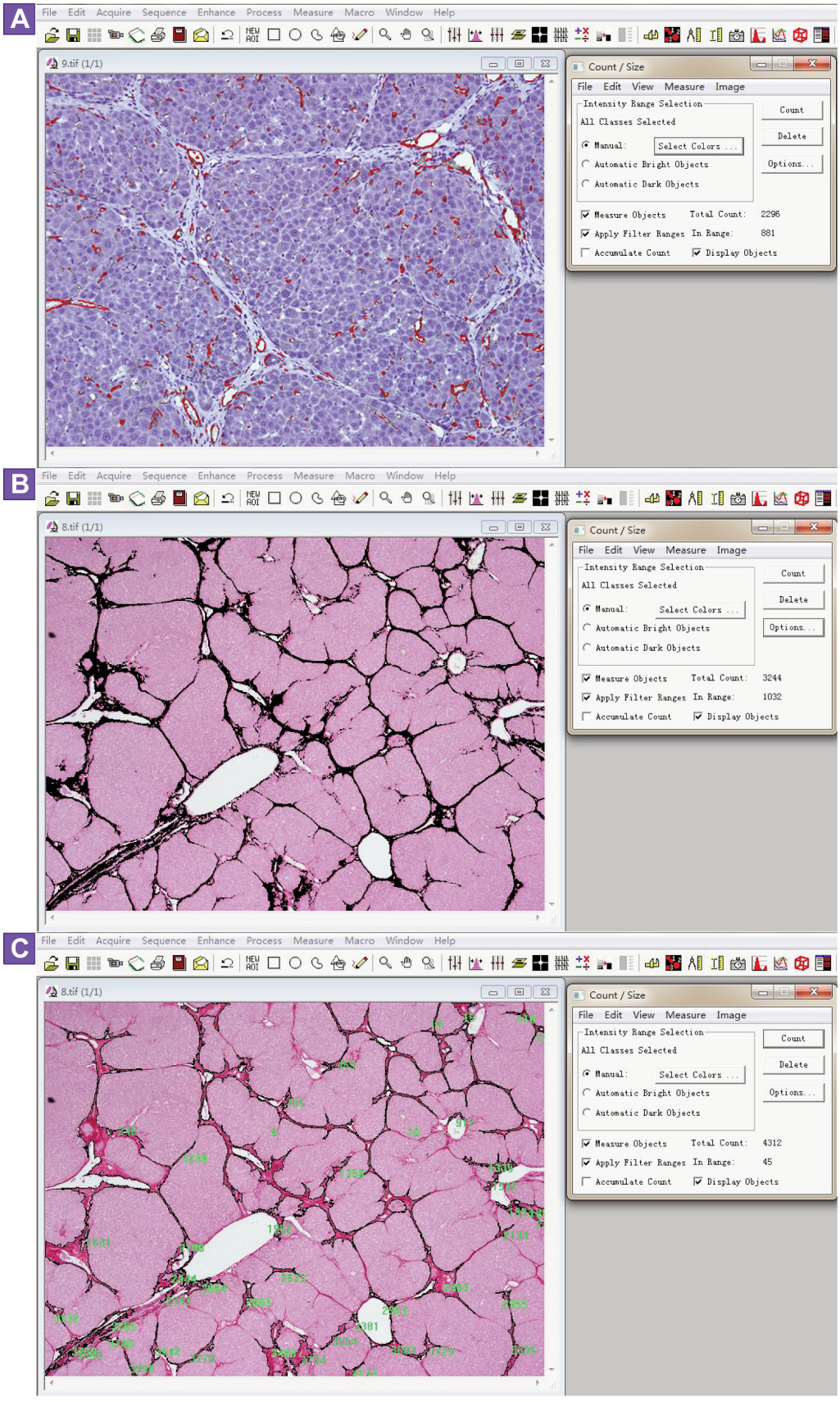

Fig. 1. Image analysis and morphometry of (**A**) microvessel density (MVD), as shown by CD34 immunostaining; (**B**) fibrosis area, as shown by Sirius Red staining; and (**C**) nodule size, as shown by maximum length (μm) of each Sirius Red-stained nodule. All images were automatically analyzed by Image Pro-plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

pressure (r=0.074, p<0.001).

Receiver operating characteristic curve analysis of MVD and fibrosis area in the discrimination of significant portal hypertension in all rats

The ROC curve was calculated from all rats. For the prediction of portal pressure ≥10 mmHg, the AUROC for MVD was 0.953 (95% CI: 0.875-1.031) and the optimal cutoff value for MVD was 18/mm^2, with 96.3% sensitivity and 93.3% specificity (Fig. 2A). Additionally, the AUROC for fibrosis area predictive of portal pressure ≥10 mmHg was 0.985 (95% CI 0.985-1.012), and the optimum cutoff value was 6.34%, with a sensitivity of 88.9% and a specificity of 100% (Fig. 2B). Micrographs of MVD and fibrosis area are shown in Fig. 3

Discussion

The process of liver cirrhosis has been shown to increase angiogenesis, both in chronic liver diseases in humans and in experimental fibrotic models in rodents (Lemos and Andrade, 2010; Coulon et al., 2011). Co-occurrence of fibrogenesis and angiogenesis in cirrhotic livers increases the resistance of the intrahepatic circulation, which plays an important role in the development of portal hypertension (Thabut and Shah, 2010). Angiogenesis in turn, is likely to significantly contribute to the perpetuation and amplification of inflammatory responses through the expression of chemokines and/or adhesion molecules and by recruiting inflammatory cells, resulting in the progression of fibrosis and the exacerbation of portal hypertension (Jackson et al., 1997). Taken together, these findings indicate that angiogenesis, fibrogenesis and portal hypertension are closely integrated.

Although MVD has been reported to correlate with the degree of liver fibrosis (Fernandez et al., 2009), its ability to discriminate portal pressure had not previously been tested. We found that MVD was significantly higher in cirrhotic rats with portal pressure ≥10 mmHg than in rats with portal pressure <10 mmHg. We also found a significant correlation between MVD and portal pressure, confirming our hypothesis that MVD is an independent histopathological determinant of significant portal hypertension. Thus, measuring MVD may have prognostic value in predicting the occurrence of portal hypertension-related complications.

Previous studies have correlated histopathological parameters on liver biopsies with portal pressure measured by HVPG in patients with cirrhosis. Those studies found that fibrosis area and nodule size were diagnostic of portal hypertension (Nagula et al., 2006; Kumar et al., 2008; Calvaruso et al., 2009; Sethasine et al., 2012; Rastogi et al., 2013). Similarly, we found that fibrosis area was an important determinant of portal hypertension, whereas MVD was superior to fibrosis area in the evaluation of significant portal hypertension. Firstly, CD34 immunostaining is more sensitive in revealing sinusoidal capillaries and isolated neo-micro vessels not recognized by staining for collagen with Sirius Red. Thus, CD34 staining may be better able than collagen staining to detect the subtle and terminal pathophysiological vascular changes associated with portal hypertension. Secondly, the formation of sinusoidal capillaries has been shown to cause portal hypertension during the early stage of liver damage,

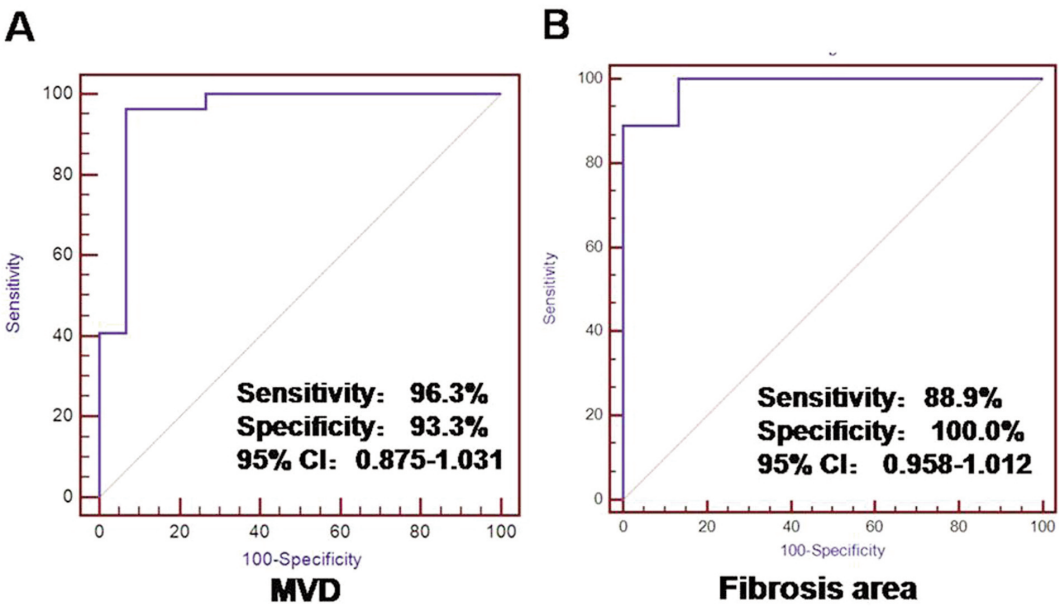

Fig. 2. Receiver operating characteristic curve analysis of (**A**) MVD and (**B**) fibrosis area as predictors of significant portal hypertension in all rats. MVD, microvessel density.

prior to collagen deposition (Zhang et al., 2004). In addition, deposition of collagen decreases during later stages of cirrhosis owing to the down-regulation of fibrogenic cytokines resulting from parenchyma distinction (Gressner et al., 2006; Kuriyama et al., 2007; Wirkowska and Paczek, 2011). In contrast, angio-architecture remodeling continues, even during the terminal stages of cirrhosis, as evidenced by the progressive increase of HVPG in cirrhotic patients. Thirdly, although fibrotic scarring is closely related to increased vascular resistance caused by structural changes, hyperdynamic splanchnic circulation, another important pathophysiologic characteristic of portal hypertension, could not be satisfactorily revealed by collagen staining (Rappaport et al., 1983; Nagula et al., 2006; Sethasine et al., 2012). Angiogenesis was not only closely associated with increased sinusoidal resistance (Thabut and Shah, 2010), but also with the formation of portosystemic collateralization (Fernandez et al., 2004, 2005, 2007) and increased intrahepatic hyperkinetic circulation (Lee et al., 1999; Sumanovski et al., 1999; Geerts et al., 2006; Moreau and Lebrec, 2006). These data, together with our findings, suggest the importance of angiogenesis in the formation and maintenance of portal hypertension, which may explain our almost perfect correlation between MVD and portal pressure.

In contrast to earlier studies, however, we observed no correlation between nodule size and portal pressure, primarily because nodule size varied considerably within each pathological section. In contrast to the large sizes of resected livers in animal models of fibrosis, enabling assays of nodule size throughout the liver, liver biopsies taken from patients are much smaller, restricting determination of nodule size due to sampling errors

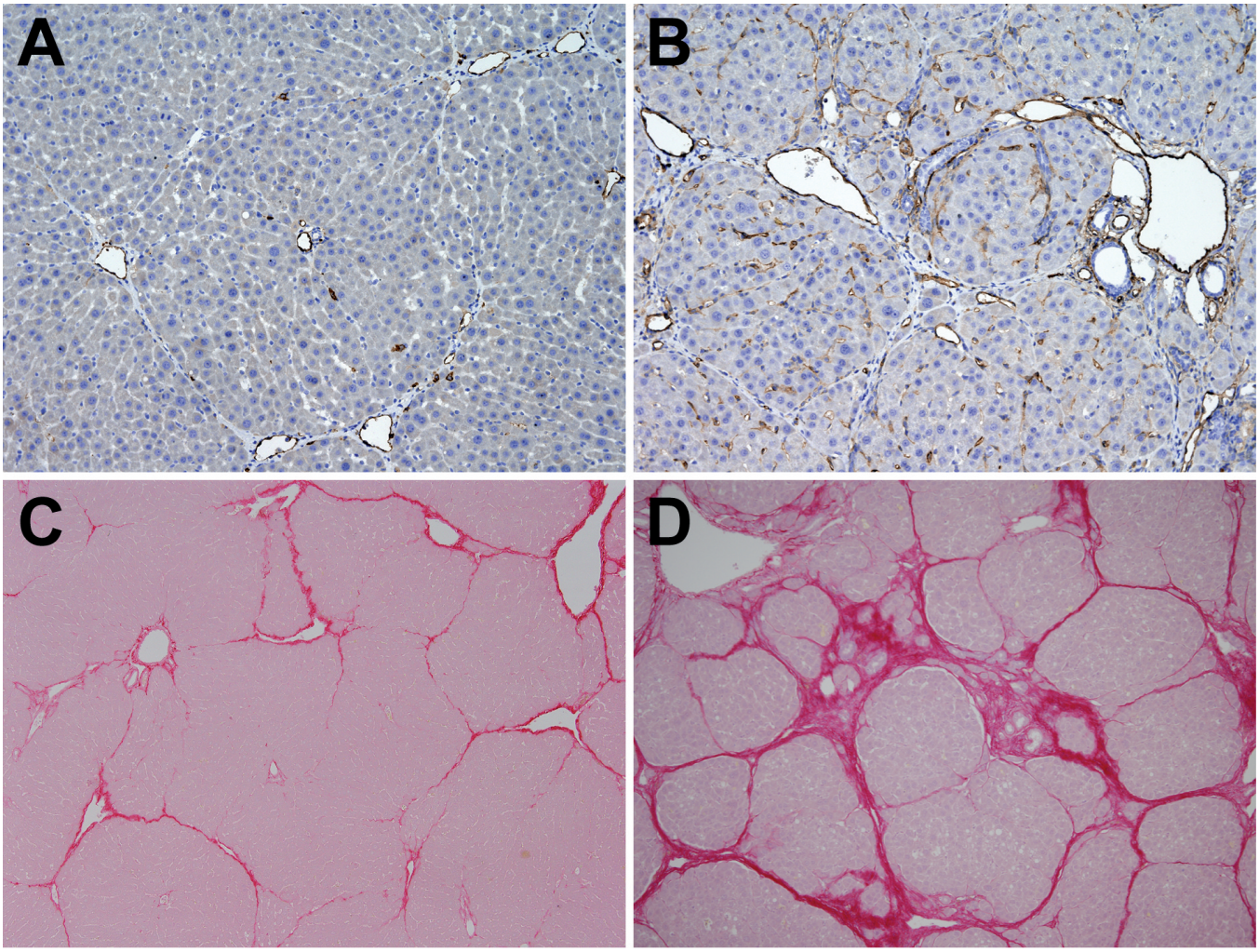

Fig. 3. Representative images in rats **(A, C)** without and **(B, D)** with significant portal pressure. In **(A)** and **(C)**, several CD34-positive vessels were detected in the fibrotic septa, with mild fibrosis starting and extending from hepatic central venules (CD_{34} immunostaining). In **(B)** and **(D)**, the cirrhotic nodules are surrounded by a dense vascular plexus (Sirius - Red). Some scattered sinusoidal endothelial cells were also positive, with the formation of small nodules. A, B, x 20; C, D, x 40

(Castera, 2008). Our findings therefore indicate that nodule size is not an appropriate histopathological parameter for the discrimination of portal hypertension. Finally, we found that spleen weight correlated significantly with portal hypertension, suggesting that measurements of spleen volume in clinical settings may have a role in the evaluation of portal hypertension.

One limitation of this study was its use of an animal model, not human patients. In fact, our preliminary results showed that the degree of angiogenesis revealed by CD34 in chronic hepatitis B patients of stage 2 and 4 step-wise increased with the severity of liver fibrosis (data not shown). At present, liver biopsy remains the gold standard for evaluating the grade and stage of chronic liver disease. Histological diagnosis of liver biopsy specimens may provide further evidence, using CD34 immunostaining to predict the likelihood of portal hypertension.

In conclusion, our results confirmed that quantification of liver fibrosis, expressed as fibrosis area, correlated with portal pressure, making this parameter useful in discriminating portal pressure. More importantly, for the first time, we showed that MVD, measured as CD34 immunostaining, was a histopathological parameter with even greater ability than fibrosis area in differentiating significant portal hypertension. Measurement of MVD in patients with liver diseases may play an important role in the evaluation of portal hypertension.

Acknowledgements. This study was supported by grants from the Beijing Nova program of China (Grant Number 2009 B45) and the National Natural Science Foundation of China (Grant Number 81000178).

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Accepted August 9, 2014