

Selective deposition of agrin  
in the microvasculature of hepatocellular carcinoma:  
aspects in pathogenesis and differential diagnosis

Doctoral (Ph.D.) theses

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## **Introduction**

In the present thesis we examine the role of agrin in the pathogenesis and differential diagnosis of hepatocellular carcinoma (HCC). Agrin is a member of the heparan sulfate proteoglycan (HSPG) protein family. HSPGs are built up of a core protein and covalently attached acidic heparan sulfate (HS) glucosaminoglycan chains. Typically, they are encountered on cell surfaces and in the extracellular matrix (ECM). The most important roles of HS chains include binding of various growth factors and cytokines and their presentation to their cognate receptors. Also, they are indispensable in biological barriers such as the renal glomerular basement membrane and the blood-brain barrier due to their high negative charge. The core proteins of HSPGs establish connections with numerous proteins on the cell surface and in the ECM. The healthy liver tissue has a relatively low HSPG content; however, in chronic liver diseases and primary liver cancers the quantity of HSPGs, similar to other ECM components, increases dramatically.

Agrin is one of the most complex members of the HSPG family. Alternative splicing of its mRNA may result in the production of either membrane-anchored or secreted isoforms. It was first identified in the neuromuscular junction as the factor that aggregates acetylcholine receptors and cholinesterase molecules in the postsynaptic membrane. Later it was also recognized that agrin directs axonal growth and synaptogenesis in the developing central nervous system. Still in the brain, but in a different context, agrin is a crucial component of the blood-brain barrier. Outside the nervous system agrin was found to be the major HSPG responsible for ultrafiltration and podocyte attachment in the renal glomerular basement membrane. In all these localizations, its extensive connections with ECM components on one hand and cytoskeleton-associated cell surface receptors on the other allow the extracellular form of agrin to form bridges between the cytoskeleton of epithelial cells and their underlying basement membranes. In addition to a purely mechanical function agrin may also initiate intracellular signaling via its membrane receptors.

Hepatocellular carcinoma accounts for 90% of primary liver cancers. It is the fifth most common malignancy worldwide, and ranks third in malignant mortality. Although HCC may arise in the structurally normal liver, the typical scenario for hepatocarcinogenesis is liver cirrhosis, the end stage of chronic liver injury. In Hungary, cirrhosis usually develops as a consequence of alcohol abuse, chronic hepatitis C, or inborn errors of metabolism. The distinction of HCC from dysplastic nodules and from atypical forms of the benign tumor hepatocellular adenoma are recurring problem situations in liver pathology. Dysplastic nodules are macroscopically apparent premalignant lesions in the cirrhotic liver. Low grade dysplastic nodules are characterized by mild histological atypia and a lack of malignization tendency, whereas high grade dysplastic nodules exhibit variable degree of atypia and are regarded as being directly precancerous. Distinguishing the latter from small HCC on the sole basis of histologic examination is often prone to subjectivity. Similarly, while typical forms of hepatocellular adenoma usually appearing in young women raise no suspect of malignancy, atypical forms often presenting at older age and/or in men may be difficult to differentiate from well-differentiated HCC.

In our earlier work we observed a strong immunohistochemical reaction in the diseased liver when using the previously poorly characterized monoclonal antibody clone 7E12. The immunostaining appeared in blood vessel walls and around ductular reaction in cirrhosis, and in the stroma of HCC. Sinusoidal walls of cirrhotic regenerative nodules remained unlabeled, while the vasculature of HCC showed an intense staining. Literature data and information available on the antibody suggested that 7E12 might react with agrin, although this was not directly stated in any of the sources.

Based on the preliminary results two assumptions were formulated: 1) the immunoreaction of 7E12 in the liver might be accounted for by agrin, a HSPG previously not detected in the liver; 2) this immunoreaction might be selective for the vasculature of HCC against sinusoids, and thus might find an application in the differential diagnostic routine. Pointing beyond the principal aim of verifying these assumptions, the question of putative cellular sources and molecular connections of

agrin in the liver was also addressed, and the possible roles of agrin in the pathogenesis and progression of hepatocellular carcinoma were briefly explored.

### **Aims**

1. Verification of the specificity of clone 7E12 for agrin.
2. Investigation of the expression of agrin mRNA and protein in the healthy liver, cirrhosis, and HCC.
3. Exploration of the putative sources and functionally relevant molecular connections of agrin in the liver.
4. Performing agrin immunohistochemistry on benign and malignant hepatocellular lesions. Addressing the applicability of agrin and CD34 immunoreactions in the evaluation of benign versus malignant character.
5. Interpretation of the deposition of agrin as a phenomenon in the context of hepatocarcinogenesis. Clarifying the relationship of agrin with the vascular remodeling process characteristic of HCC.
6. Extending the investigations to an experimental rodent model for the purpose of future *in vivo* experimentation.

## **Methods and materials**

- Immunoprecipitation and mass spectrometry analysis of HSPG isolated from bovine kidney cortex
- Western blot on protein isolates from healthy human liver, cirrhosis, and HCC
- Real time RT-PCR on human samples, healthy rat liver, and the liver of rats subjected to simultaneous cirrhosis/HCC induction
- Immunofluorescent and double immunofluorescent colocalization experiments on human samples, healthy rat liver, the liver of rats subjected to simultaneous cirrhosis/HCC induction, and cultured cells
- Immunohistochemistry on human pathologic and forensic specimens
- Semi-quantitative evaluation of agrin and CD34 immunoreactions; comparing the results with histopathologic diagnoses; devising an immunohistochemistry-based diagnostic protocol
- *In situ* hybridization for agrin using digoxigenin-labeled RNA probe on human cirrhosis and HCC

## **Results**

### *1. Verification of the specificity of clone 7E12 for agrin*

By double immunofluorescent reactions we demonstrated the colocalization of the immunoreactions 7E12 with anti-laminin as well 7E12 with a commercially available polyclonal anti-agrin antibody. Subsequently, immunoprecipitation was performed using 7E12 and magnetic beads from the proteoglycan extract of bovine kidney cortex, the immunogen used for the development of 7E12. The isolated proteoglycan component was subjected to mass spectrometry analysis. Three tryptic fragments (FGALCEAETGR, CEPGFWNFR, IFFVNPAPPYLWPAHK) were identified that correspond to *Bos taurus* *Similar to agrin*, the bovine homologue of agrin.

### *2. Agrin mRNA and protein expression in the healthy liver, cirrhosis, and HCC*

Frozen specimens from healthy human liver (14), cirrhosis (13), and HCC (16) were used for RNA isolation and subsequent real-time RT-PCR to determine the relative expression of agrin. The quantity of agrin mRNA was significantly, approx. 5-fold higher in both HCC and cirrhosis when compared to the normal liver ( $p < 0.0001$  and  $p = 0.0006$ , respectively). Accordingly, no significant difference was measured between HCC and cirrhosis. The protein expression of agrin was determined using proteoglycan isolates of one representative specimen each from normal liver, cirrhosis, and HCC. No signal was detected in the normal liver sample, whereas strong smears above 400 kDa corresponding to the high molecular weight of glycosylated agrin were seen in the cirrhotic and HCC samples.

### *3. Putative sources and functionally relevant molecular connections of agrin in the liver*

Agrin colocalized with smooth muscle alpha actin (SMA) in the muscular layer of blood vessel walls in both the human and the rat liver. Partial colocalization of agrin and SMA was also seen in the vasculature of HCC in both species. Myofibroblasts

isolated from the liver of healthy rats and, being activated by *ex vivo* cell culturing conditions, expressing SMA produced agrin on the mRNA and protein level. However, intense immunoreaction without associated SMA-positive cells was observed in the basement membrane of ductular reaction epithelium in the human and rat cirrhosis. A faint agrin immunostaining was attributed to transitional phenotype cells at various stages of differentiation from bile ductular epithelium to hepatocytes. These cells were located in the marginal zone of cirrhotic regenerative nodules adjacent to ductular reaction and expressed cytokeratin-7 to variable extent. In the aforementioned localizations the mRNA of agrin was also detected by *in situ* hybridization; however, this method needs further technical improvement. Finally, pseudoacinar-trabecular arrays of well-differentiated hepatocellular tumor cells (in adenomas and highly differentiated HCCs) were occasionally surrounded by agrin-containing basement membranes with no association of biliary or vascular markers.

Agrin colocalized with the proangiogenic basic fibroblast growth factor in the stroma of HCC. Moreover, epithelial cells lying on agrin-positive basement membranes such as endothelial cells and the epithelium of ductular reaction exhibited  $\alpha_v$ -integrin immunopositivity. Alpha(v)-integrins are also known as agrin receptors.

#### *4. Agrin immunohistochemistry on benign and malignant hepatocellular lesions. The applicability of agrin and CD34 immunoreactions in differential diagnosis*

One hundred and thirty-two specimens (25 cirrhotic livers, 10 cases with focal nodular hyperplasia [FNH], 8 large regenerative nodules [LRN], 23 low-grade dysplastic nodules [LGDN], 7 high-grade dysplastic nodules [HGDN], 30 hepatocellular adenomas [HA], 8 small HCCs, and 21 HCCs) were subjected to agrin immunohistochemistry and semi-quantitative evaluation. A 5-grade scoring system (0 – 4+) was applied. Strong (3-4+) immunoreactions were primarily observed in the sHCC and HCC groups. Strong labeling occurred only occasionally in hepatic adenomas and HGDNs, and no immunostaining stronger than 2+ was encountered in the remaining groups.

The results of semi-quantitative evaluation were used to examine the usefulness of agrin immunoreaction in the benign-malignant problem situation. Cirrhotic and FNH samples were excluded as being irrelevant to the question of malignant character. Immunoreactions of 2+ and weaker were overwhelming in the benign groups (LRN, LGDN, HGDN, HA), in sharp contrast with the dominance of 3-4+ reactions among sHCC and HCC samples. Hence, classifying 0-2+ cases as 'immunohistochemically benign' or 'IHC-benign' and 3-4+ cases as 'IHC-malignant' and comparing the grouping with that based on histopathologic diagnoses, a sensitivity of 93.1% but a specificity of only 88.2% was obtained. Poor specificity was caused by ranking many histopathologically benign cases with agrin 3+ immunoreaction in the 'IHC-malignant' group. Specificity could be improved by also evaluating CD34 immunoreactions in agrin 3+ cases and regarding only CD34 4+ cases as 'IHC-malignant'. With this extension, specificity was increased to 92.6%.

#### *5. The relationship of agrin with the process of hepatocarcinogenesis. Possible causes of agrin production*

In the cirrhotic regenerative nodules no clear correlation was seen between mitotic activity assessed by PCNA immunoreaction and the appearance of agrin. Nor was the presence or absence of markers that reflect changes in vascular phenotype such as SMA, CD34, and claudin-5 unequivocally correlated with the presence or absence of agrin. These observations indicate that neither increased proliferation of hepatocytes nor activation of mesenchymal cells and the switch in endothelial phenotype (collectively referred to as 'capillarization') may alone account for triggering agrin production.

#### *6. Agrin expression in the healthy and damaged, cirrhosis/HCC-induced rat liver*

The livers of four rats subjected to simultaneous cirrhosis/HCC induction were compared to the healthy rat liver. In the treated animals multifocal HCC developed in a cirrhotic background. Immunohistochemical and immunofluorescent localization of agrin conformed well to human observations in both the healthy and the diseased rat liver. Agrin mRNA expression of the healthy vs. treated livers was also compared. The



multifocal nature of HCC and the microscopic size of the malignant nodules prevented us from evaluating cirrhosis and HCC separately. Instead, multiple specimens were taken from the treated livers to assess intra-organ heterogeneity. Despite differences between samples from the same organ 1.8- to 4-fold elevated expression values were measured in all treated specimens.

## Conclusions

1. The specificity of the monoclonal antibody 7E12 for agrin has been verified.
2. Agrin mRNA and protein have been proven to accumulate in cirrhosis and HCC.
3. As the putative sources of agrin in the liver, vascular smooth muscle cells, activated mesenchymal cells, bile duct and reactive ductular epithelium, transitional phenotype cells differentiating from ductular reaction to hepatocytes, and well-differentiated hepatocellular tumor cells have been proposed. Agrin in the liver is supposed to interact with basic fibroblast growth factor and  $\alpha_v$ -integrin receptors, allowing to envision a role for agrin in the formation of ductular reaction and in neoangiogenic processes.
4. By comparing benign and malignant parenchymal lesions a remarkable specificity of agrin immunoreaction for the microvasculature of HCC has been proven, which forecasts that agrin immunohistochemistry may find an application in the liver pathology routine.
5. The direct causes of agrin production have remained unclear. The appearance of agrin failed to clearly correlate with either proliferative activity or capillarization.
6. The deposition of agrin in liver cirrhosis and HCC in the rat has been found analogous with the human example. Therefore, a rodent model is considered suitable for future *in vivo* investigations concerning the role of agrin in chronic liver diseases.

## **List of publications**

*In the topic of the doctoral theses:*

**Tatrai P**, Dudas J, Batmunkh E, Mathe M, Zalatnai A, Schaff Z, Ramadori G, Kovalszky I. Agrin, a novel basement membrane component in human and rat liver, accumulates in cirrhosis and hepatocellular carcinoma. Lab Invest. 2006 Nov;86(11):1149-60.

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