Expression of agrin in primary liver tumors

Ph.D. Thesis Synopsis

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1. INTRODUCTION

The incidence of primary liver tumors is increasing. According to the World Health Organization, at least 550,000 people die each year due to primary liver tumors, therefore, hepatocellular carcinoma (HCC) as the most frequent type of primary malignant liver tumors is one of the leading cancers in the world. In some parts of the world, such as tropical Africa and some parts of Asia, as well as in Mongolia HCC is one of the most common cancers. Chronic viral hepatitis can induce cirrhosis, which increases the risk of HCC. Recent insight into the molecular mechanisms leading to HCC development has been provided by the identification of major genetic abnormalities revealed by genome-wide allelotype studies and molecular cytogenetic analysis. However, not all processes are clearly understood.

The extracellular matrix (ECM) provides a dynamic microenvironment surrounding cancer cells that contributes to growth and phenotypic transformation during tumorigenesis by acting at both cellular and molecular levels. Understanding the mechanisms that regulate the interactions between cancer cells and ECM may thus create new paradigms by which targeted therapeutic interventions for this lethal disease could be developed. Heparan sulphate proteoglycans (HSPG) are ubiquitous, being present on the cell surface or as insoluble components of the ECM. HSPGs are known to function as co-factors in cell–cell adhesion, in linking cells to ligands in the extracellular matrix, in the binding and activation of cellular growth factors and in the control of extracellular proteolysis during thrombus formation and cell invasiveness. All of these functions, for which the heparan sulfate (HS) moiety of the proteoglycans appears to be critical, may be relevant to the initiation or promotion of malignant cellular transformation, tumor growth, tumor cell adhesion, invasiveness and metastasis.

Many functions of the basement membrane HSPG, agrin, have been identified in the literature, but its role in carcinogenesis is unknown. To our current knowledge, HSPGs (e.g. perlecan) influence the processes of cellular growth, adhesion, migration, and matrix turnover during embryogenesis, regeneration and carcinogenesis. On the basis of all these, agrin seems to be able to participate in these processes.

Since interactions between cells, the matrix, and growth factors play a major role in liver carcinogenesis, the aim of this study was to investigate whether the expression and distribution pattern of agrin is altered in human primary liver tumors.

2. The aim of the study

1. To examine the expression level of agrin mRNA and protein in normal human liver.

2. To examine the differences in the expression and localization of agrin in cirrhosis compared with normal liver.

3. To study the differences in the expression of agrin in hepatocellular carcinomas (HCC) compared with normal liver.

4. To investigate the differences of agrin expression between HCC and cholangiocarcinomas (CC).

5. To investigate the expression of agrin in benign liver tumors such as hepatocellular adenoma (HCA) and focal nodular hyperplasia (FNH).

6. To study the angiogenesis related distribution of agrin in liver tumors.

7. To examine how the differentiation of tumors (HCC, CC) corresponds to the expression of agrin protein.

3. MATERIALS AND METHODS

3.1. Human tissue specimens

Total of 104 cases of surgically resected liver tumors with corresponding nontumorous liver tissue were studied, which included 35 HCC, 35 CC, 14 HCA and 20 FNH cases. Ten normal liver samples were collected from patients who died in accidents or non-liver related diseases. The material was collected with the permission of the Regional Ethical Committee of the Semmelweis University (#172/2003, # 2/2004).

The surgically resected tumor samples with their surrounding nontumorous liver, as well as the normal livers were fixed in buffered formalin and snap frozen in liquid nitrogen or fixed in RNA later (Sigma, Saint Louis, Missouri, USA) and stored at -80˚C until further analysis. For RNA isolation, RNA later-fixed materials were used. The diagnosis was established on sections processed for routine histopathology, stained by hematoxylin and eosin (HE), PAS, picrosirius red, Shikata's orcein.

3.2. Methods

3.2.1.Study of the protein expression

Immunohistochemistry and immunfluorescence

The immunohistochemical reactions were carried out on paraffin embedded and frozen sections. For the detection of antigens the following antibodies were used: anti-agrin, -laminin, -CD34, -Ki67, -CK7. For the detection of agrin the signal amplification was enhanced by biotinylated-tyramine according to the protocol. For all sections 3-amino-9-ethyl-carbazole (AEC) or diamino-benzidine (DAB) was used as chromogen and Mayer's hematoxylin as nuclear counterstain.

The immunofluorescence reactions were carried out on frozen sections. For the detection of antigens the following antibodies were used: agrin, laminin, α_v-integrin, CD31, bFGF and smooth muscle α-actin. The immunfluorescence reactions were examined by confocal laser-scanning microscopy (Bio-Rad MRC-1024 system, Bio-Rad, Richmond, CA, USA).

Negative controls for nonspecific binding, incubated with secondary antibodies only, were processed and revealed no signal.

Morphometry

The immunohistochemical reactions detecting agrin were photodocumented using light microscopy (200X magnification, Olympus BX microscope). The immunofluorescence reactions were examined by confocal laser-scanning microscopy. Digital images showing immunoreactivity of agrin were quantified using Leica QWin software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK).

Detection of agrin protein expression by Western blotting

Proteoglycans (PGs) were isolated from equal weights of frozen material according to the modified method of Lyon and coworkers.

Aliquots of PG preparations in SDS loading buffer were loaded and separated on gradient gels. Electrophoresis was followed by transfer onto nitrocellulose filters. Membranes were blocked with 5%(w/v) BSA in TBS (pH 7.6). Immunoblot analysis to detect agrin was performed with 1:500 dilution of the primary antibody diluted in 5% BSA overnight at 4ºC. Blots were washed and incubated with secondary horseradish peroxidase-conjugated goat anti-mouse IgG (SIGMA). Bands were visualized by enhanced chemiluminescence (ECL, Amersham).

3.2.2.Analysis of mRNA expression

RNS isolation from RNA later fixed samples

RNA was extracted from 200 mg liver samples fixed in RNA later (Sigma) with TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA pellet was washed once in 70% ethanol, dried and resuspended in 50 µl of RNase free water and kept at -80˚C until further use. The integrity of total RNA was verified by gel electrophoresis.

Real-time RT-PCR

An aliquot of total RNA was reverse transcribed with M-MuLV reverse transcriptase by using random hexamers. Real-time RT-PCR was performed by using agrin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA specific primers.

Specific real-time PCR reactions to detect agrin and GAPDH were carried out in Sybr Green PCR Master Mix (BIO-RAD 1708882) with 500 nM of each primers using the ABI Prism 7000 sequence detection system (Applied Biosystems). After initial denaturation at 95 °C for 2 min, 40 cycles were performed at 95°C for 20 seconds, at 60 °C for 30 seconds and at 72°C for 30 seconds.

Evaluation of the data for relative quantification to reveal statistical differences between the groups to be compared was carried out with Relative Expression Software Tool (REST) by Pairwise Fixed Reallocation Randomisation Test. Relative quantification method was utilized for data analysis by using GAPDH as reference gene.

3.2.3. Statisical analysis

The statistical analysis was carried out by GraphPad Prism software version 2.01 (GraphPad Software, Inc. San Diego, CA, USA). The Mann-Whitney U test was used to compare the expression of agrin in the different groups, p value < 0.05 was accepted as being significant.

4. Results

4.1. Immunohistochemical detection of agrin

4.1.1 Immunolocalization of agrin in normal liver and cirrhotic surrounding liver

Immunohistochemistry showed positivity around the bile ducts and the blood vessels within the portal area in normal liver; however, no expression within the hepatic acini along the sinusoids was found. Agrin protein expression was not detected along the sinusoids in cirrhotic nodules, however, strong positivity was found around proliferating bile ducts and blood vessels in fibrous septa.

4.1.2 Immunohistochemical detection of agrin in hepatocellular carcinoma

In HCCs, strong agrin expression was seen, mostly localized along the neovascular basement membrane. There was no intracellular agrin localization in the tumor cells. CD34 expression corresponded well with the location of agrin expression.

4.1.3 Co-localization of agrin with other basement membrane components

Agrin obviously co-localized with the laminin in connective tissue septa of the HCCs. Basic fibroblast growth factor (bFGF) was also found in all agrin-positive basement membranes. Agrin and α_v-integrins, given their co-localization or close spatial proximity, may in principle interact in bile ducts, blood vessels and in the tumor stroma.

4.1.4 Immunolocalization of agrin in cholangiocarcinoma

In cholangiocarcinoma (CC), agrin was strongly expressed in the basement membrane surrounding the tumor cell-composed glandular and trabecular structures, in both the well- and moderately differentiated cases. In contrast, the expression was fragmented, decreased, and less intense or even absent in the poorly differentiated areas of the tumors and at the sites of invasion into the non-tumorous surrounding tissue. There was no intracellular localization of agrin in carcinoma cells and stromal myofibroblasts.

The expression of CD34 endothelial marker corresponded well with the location of agrin expression in the stromal vessels of the tumor, however, it was not found in the basement membrane surrounding the tumor cell-formed glandular and trabecular structures on consecutively cut serial sections.

4.1.5. Quantitative analysis of agrin positive reaction

Quantitative analysis of the areas positive for agrin by immunohistochemistry in the percentage of total investigated area resulted in the following mean values ± s.e: 0.22 ± 0.029% for normal livers, 1.55 ± 0.121% for HCC and 3.72 ± 0.277% for CC. These results show that significantly ($p < 0.0001$) more agrin was detected in the CCs as compared with the HCCs. Both HCCs and CCs contained significantly ($p < 0.0001$) more agrin in comparison to normal livers. In well and moderately differentiated CCs, the percentage of immunopositive areas was significantly higher ($p< 0.0001$; $p< 0.0001$) when compared with the poorly differentiated cases. There was no significant difference between well- and moderately differentiated CCs.

4.1.6. Immunolocalization of agrin in hepatocellular adenoma

We investigated protein expression of agrin in 14 hepatocellular adenoma (HCA) by immunohistochemistry. The agrin expression showed difference within the tumors. In some areas the expression of agrin showed strong positivity, but elsewhere it was negative. The expression of CD34 endothelial marker corresponded well with the location of agrin expression along the sinusoids of HCA.

4.1.7. Immunolocalization of agrin in focal nodular hyperplasia

The typical lesion of focal nodular hyperplasia (FNH) is a benign tumor-like mass characterized by hepatocytic nodules separated by fibrous bands and by central stellate scar. In FNH intensive agrin expression was detected in the basement membrane of the proliferated bile ducts, however, no agrin expression was observed along the sinusoids**.**

4.2. Western blotting analysis of agrin in proteoglycan extracts

PG extracts from representative samples of CCs and HCCs were unequivocally positive for agrin, while the PG extract from the normal liver showed negative reaction on Western blot analysis. Given the equal initial amounts of material used for PG isolation and subsequent Western blotting the CC samples revealed markedly higher expression of agrin compared with HCCs.

4.3. Real time RT-PCR analysis of agrin mRNA expression

The expression of agrin mRNA in the CC sample group was significantly, 10.75 folds upregulated in comparison with the normal liver group. Agrin mRNA also showed a significant 3.4 fold upregulation in the CC group when compared with the HCCs. In the HCC samples, agrin was upregulated 2.3 folds as compared with the normal liver, however, the difference was not statistically significant.

The results of immunohistochemistry and real-time PCR seem to positively correlate: both protein and mRNA expressions of agrin were significantly higher in CCs as compared with normal livers and HCCs, whereas agrin expression was higher in the HCCs compared with the normal livers.

5. CONCLUSIONS AND ORIGINAL FINDINGS

1. In the normal liver agrin protein expression was detected exclusively in portal tracts: around the bile ducts and the blood vessels, however, no expression was found within the hepatic acini along the sinusoids.

2. In cirrhotic liver strong positivity of agrin expression was detected around proliferating bile ducts and blood vessels in fibrous septa, while the sinusoids were negative.

3. In HCCs, strong agrin expression was seen, mostly localized along the neovasculature of tumors.

4. The expression of CD34 endothelial marker corresponded well with the localization of agrin expression in the stromal vessels of HCCs.

5. In CCs, agrin was strongly expressed in the basement membrane surrounding the tumor cell-composed glandular and trabecular structures, in both well- and moderately differentiated cases, in which CD34 was negative.

6. In contrast, the agrin expression was fragmented, decreased, and less intense or even absent in the poorly differentiated areas of the tumors and at the sites of invasion into the non-tumorous surrounding tissue.

7. There was no intracellular localization of agrin in carcinoma cells and stromal myofibroblasts.

8. The results of immunohistochemistry and real-time PCR seem to positively correlate: both protein and mRNA expressions of agrin were significantly higher in CCs as compared with normal livers and HCCs, whereas agrin expression was higher in the HCCs compared with the normal livers.

9. In HCA, agrin expression showed intratumoral differences. In some areas agrin showed strong expression along the sinusoids, while elsewhere it was negative.

10. In FNH, intensive agrin expression was detected in the basement membrane of the proliferating bile ducts, while no agrin expression was observed along the sinusoids.

6. PUBLICATION LIST

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