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論　文　名	Pathological Studies on Myofibroblast Properties in Thioacetamide-Induced Rat Liver Fibrosis and Cirrhosis with Special Emphasis on Glial Fibrillary Acidic Protein Expression （グリア線維性酸性蛋白質の発現に重点を置いたチオアセトアミド誘発ラット肝線維化と肝硬変における筋線維芽細胞の特性に関する病理学的研究）
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論文要旨

Introduction

Liver is one of the indispensable organs in the body. Despite its immense regenerative capability, fibrosis of the liver and its end stage, cirrhosis, represent a massive health care burden worldwide. Fibrosis itself is a wound healing response; however, derangement of this physiological response leads to pathological scarring. Following transient injury, liver can generally reconstitute itself entirely. However, chronic repetitive injury causes persistent liver fibrosis, ultimately culminating in cirrhosis with multiple complications such as portal hypertension, hepatic encephalopathy and hepatocellular carcinoma (HCC). Hepatitis C and B viral infection, nonalcoholic steatohepatitis, alcohol abuse and various autoimmune diseases are the

major causes of cirrhosis. No drug has yet been developed as an effective anti-fibrotic agent, and currently orthotropic liver transplantation is the only effective treatment for end-stage-liver disease. Therefore, understanding the pathophysiology behind liver fibrosis is important to develop new therapeutic targets.

Excessive deposition of extracellular matrix (ECM) such as collagens is the key event in fibrosis/cirrhosis; myofibroblasts are the source of ECM. They are intermediate in nature between fibroblasts and smooth muscle cells. Additionally, myofibroblasts may contribute to hepatic progenitor cell behavior, pseudo-lobule formation and HCC. Myofibroblasts express different sets of cytoskeletal proteins such as vimentin, desmin, and α-smooth muscle actin (α-SMA) during differentiation. Hepatic stellate cell (HSC)-derived myofibroblasts exhibit glial fibrillary acidic protein (GFAP) in liver fibrosis, a type III intermediate filament protein specific generally for astroglia. Furthermore, recent studies indicate that GFAP is expressed in biliary cells as well as in liver progenitor cells. The properties of GFAP-expressing cells in injured liver are elusive.

Therefore, to analyze the characteristics of myofibroblast development in liver fibrosis with particular relation to GFAP expression, a series of studies were conducted using thioacetamide (TAA; hepatotoxicant causing centrilobular injury)-induced acute rat liver injury/fibrosis and cirrhosis. In Chapters 1 and 2, the author immunophenotypically investigated GFAP-expressing HSCs and myofibroblasts in TAA-induced acute liver injury/fibrosis and cirrhosis, respectively. In Chapters 1 and 2, additionally, factors influencing myofibroblast development were analyzed at mRNA level. In Chapter 3, the participation of GFAP-expressing cells in ductular reaction in relation to myofibroblast development and HCC development was analyzed. Based on the properties of GFAP-expressing cells, the pathogenesis of TAA-induced lesions could be partly clarified.

Chapter 1: Characterization of glial fibrillary acidic protein (GFAP)-expressing hepatic stellate cells and myofibroblasts appearing in thioacetamide (TAA)-induced acute rat liver injury and fibrosis

Section I: GFAP expression in normal rat tissues

To identify the distribution of GFAP-expressing cells in normal rat tissues, major organs including the liver were collected from 10-week-old normal F344 rats. In addition to astrocytes and Schwann cells, GFAP expression was seen in extra-neural tissue cells such as HSCs, pancreatic stellate cells, and renal interstitial cells, as well as epithelial cells of bile ducts and lens. Interestingly, GFAP-positive cells had similar morphology

(stellate) and some of them are distributed in relation to vessels as the blood-tissue interface.

Section II: Myofibroblast phenotypes and their mutual association with special emphasis on GFAP-expressing cells in TAA-induced acute rat liver injury and fibrosis

To investigate the myofibroblast phenotypes, F344 rats were injected with TAA (300 mg/Kg BW, once, ip). Liver samples were collected on post-single injection (PSI) days 1, 2, 3, 5, 7, and 10. Fibrotic lesion after hepatocyte necrosis in the centrilobular areas was seen on PSI days 1-5, with the peak on PSI day 3, and thereafter, healed by PSI day 10. Myofibroblats expressed such cytoskeletons as α-SMA, vimentin, and desmin in a varying degree; GFAP was expressed in these myofibrobalsts, peaking on PSI day 3, of which pattern was confirmed at mRNA level. Double staining indicated that GFAP-expressing HSCs reacted also to vimentin and desmin, but not α-SMA in control livers, whereas GFAP-positive myofibroblasts concomitantly expressed vimentin, desmin and α-SMA, showing proliferating activity. Interestingly, GFAP-expressing myofibroblasts reacted to stem cell markers such as nestin (for neuroepithelial stem cells) and A3 (for mesenchymal stem cells), indicating possible origin of myofibroblasts in the stem cell lineage. During the healing process, exclusively, α-SMA-positive myofibroblasts underwent apoptosis, indicating disappearance of myofibroblasts. mRNAs of TGF-ß1, TNF-α, PDGF-ß, MMP-2 and TIMP-2 increased in agreement with hepatic injury and subsequent fibrosis, indicating their participation to the phenotypical alteration of myofibroblasts.

Chapter 2: Immunophenotypical analysis of glial fibrillary acidic protein (GFAP)-expressing hepatic stellate cells and myofibroblasts in rat liver cirrhosis induced by repeated injections of thioacetamide (TAA)

To investigate myofibroblast properties in cirrhosis, F344 rats were repeatedly injected with TAA (100 mg/Kg BW, twice a week for 25 weeks, ip). Liver samples were collected at post-first injection (PFI) weeks 5, 10, 15, 20 and 25. Hepatocyte degeneration was evident in the perivenular areas at PFI week 5 with collagen deposition. With time, fibrous bridges were gradually formed between the central veins and/or Glisson's sheath, separating parenchyma into complete pseudolobules at PFI week 15 onwards. Myofibroblasts expressing α-SMA, vimentin, and desmin were developed particularly in fibrous septa, with the peak at PFI week 15. GFAP-expressing myofibroblasts simultaneously expressed vimentin, desmin or α-SMA in a varying degree and some of them reacted to nestin, A3 and Thy-1 (mesenchymal stem cells). GFAP-expressing

myofibroblasts underwent either proliferation or apoptosis. mRNA expressions of TGF-ß1, TNF-α, PDGF-ß, MMP-2 and TIMP-2 were related to myofibroblast development. In cirrhosis, GFAP-expressing myofibroblasts might have more dynamic properties, showing various cytoskeletons and proliferation or apoptosis; they might be recruited consistently from the stem cell lineage.

Chapter 3: Glial fibrillary acidic protein (GFAP)-expressing cells in ductular reaction in rat liver cirrhosis induced by repeated injections of thioacetamide (TAA)

Section I: Histopathology of ductular reaction in TAA-induced rat liver cirrhosis

Ductular reaction is a pattern of proliferation of the most terminal branches of the biliary tree, which contain hepatic progenitor cells with bipotential towards hepatocytes or bile duct epithelia. Additionally, ductular cells could give rise to myofibroblasts and HCC. Characteristics of ductular reaction were analyzed using cirrhosis model. Marked ductular cell proliferation, demonstrable by CK19 (for hepatic progenitor cells) immunohistochemistry, was seen along the developing fibrous septa. The ductular reaction was related with progressive fibrosis.

Section II: Characterization of ductular cells in terms of epithelial to mesenchymal transition (EMT) or hepatocellular carcinoma (HCC)

Hepatic progenitor cells expressing GFAP in ductular reaction are closely associated with myofibroblasts. Using cirrhosis model, myofibroblast development from ductular cells via EMT was investigated. CK19- and GFAP-expressing cells in the ductular reaction co-expressed E-cadherin, however, did not react to α-SMA (mature myofibroblasts), denying EMT of ductular epithelia. Interestingly, GFAP- and CK19-positive ductular cells reacted to α-fetoprotein (AFP, for HCC) and ß-catenin (for Wnt/ß-catenin signaling relating to neoplastic proliferation) with occasional nuclear localization. Furthermore, ductular reaction was related to increased expression of hepatocarcinogenesis-related factors (Wnt2, Wnt4 and glypican-3); these findings indicated that ductular reaction-constituting cells might be associated with carcinogenesis. Collectively, the ductular reaction in cirrhosis might have roles in liver regeneration or converse tasks as cancer stem cells.

Conclusions

Myofibroblasts play important roles in liver fibrosis, showing various cytoskeletons.

Their properties were analyzed with special emphasis on GFAP expression in TAA-induced acute rat liver injury/fibrosis and cirrhosis.

1. In normal rat tissues, GFAP-expressing cells were seen in astrocytes and Schwann cells, as well as extra-neural cells such as HSCs and biliary epithelia.

2. In both TAA-induced acute liver injury/fibrosis and cirrhosis, GFAP-expressing HSCs gave rise to myofibroblasts, expressing different cytoskeletons such as vimentin, desmin and α-SMA in a varying degree. In the acute injury, the development was transient, whereas the appearance was consistent in cirrhosis, indicating the close relation with progressive fibrosis.

3. Interestingly, GFAP-expressing myofibroblasts reacted to stem cell markers such as nestin, A3 and Thy-1 (only in cirrhosis), indicating that, along with direct participation of HSCs, myofibroblasts might be recruited from the stem cells.

4. In both acute liver injury and cirrhosis, mRNA expressions of fibrosis-related factors such as TGF-ß1, TNF-α, PDGF-ß, MMP-2 and TIMP-2 corresponded to myofibroblast development, indicating importance of these factors for alteration of myofibroblasts.

5. In acute liver injury/fibrosis, myofibroblasts expressing α-SMA underwent apoptosis for healing and GFAP-expressing myofibroblasts in cirrhosis showed either proliferation or apoptosis, indicating their dynamic participation in progressive fibrosis.

6. In cirrhosis, GFAP-expressing hepatic progenitor cells in the ductular reaction reacted to CK19 and E-cadherin, but not α-SMA, denying EMT. Interestingly, the progenitor cells were positive for AFP and ß-catenin and the ductular reaction was related to increased expression of hepatocarcinogenesis-related factors (Wnt2, Wnt4 and glypican-3), indicating that the ductular reaction-constituting cells might be associated with carcinogenesis.

7. The present studies provide useful information on myofibroblast properties for the pathogenesis of hepatic fibrosis and cirrhosis, based on GFAP expression, which may lead to possible therapeutic strategies against this intractable disease.

審査結果の要旨

肝臓は生体において代謝の中心的役割を果たす重要な器官である。それゆえに、慢性的な肝疾患は、ヒトや動物に重篤な健康障害をもたらす。特に、肝炎ウイルス感染、アルコール中毒、メタボリック症候群としての非アルコール性脂肪変性、化学物質による中毒などによる肝細胞傷害では、軽微であれば修復性の線維化が生じ回復するが、持続すれば難治性の肝硬変へと進展する。さらに、増悪すれば肝硬変から肝癌の発症に至る。肝線維化から肝硬変の進行を制御する有効な治療法は未だない。

肝線維化/肝硬変の形成には、コラーゲンなどの細胞外基質を異常に産生する筋線維芽細胞が重要な役割を演じる。筋線維芽細胞は、線維芽細胞と平滑筋細胞の双方の性状を有し、その形成過程においてビメンチン、デスミンやα-平滑筋アクチン(SMA)などの細胞骨格を発現する。グリア線維性酸性蛋白質(GFAP)も細胞骨格のひとつで、脳のアストロサイトに特異的に発現するとされていたが、正常な肝臓での肝星細胞にも発現することが見出された。しかし、肝線維化/肝硬変における GFAP 発現細胞の特性や病変形成における役割については未だ明らかにされていない。この研究では、肝毒性物質であるチオアセトアミド(TAA)誘発の急性肝傷害と TAA 連回投与による肝硬変のラットモデルを作製し、GFAP 発現細胞の動態と役割を、主に免疫組織化学的手法により解析している。

第1章では、TAA 単回投与による急性肝傷害における GFAP 発現細胞の動態を検討している。このモデルでは、投与後 1-2 日に小葉中心領域の肝細胞傷害部位に線維化が生じ、3 日にピークとなり、その後 10 日までに傷害組織は修復された。筋線維芽細胞は、線維化の形成に伴い徐々に増加し、ビメンチン、デスミンそして SMA などの細胞骨格をさまざまな割合で発現するとともに、GFAP を共発現することが分かった。修復性の線維化に係わる筋線維芽細胞は、既存の肝星細胞に由来することが示された。また、筋線維芽細胞の出現に一致して線維化誘導因子である TGF-β1、TNF-α、PDGF-β や MMP-2 が増加することを明らかにした。

第 2 章では、TAA を 25 週間週 2 回投与することで生じる進行性の線維化（肝硬変）に出現する筋線維芽細胞の特性を解析している。このモデルでは 15 週以降に線維性架橋が形成され、その結果偽小葉が生じた。筋線維芽細胞は架橋部位に集簇して出現し、第 1 章と同様に、GFAP に加え、ビメンチン、デスミンや SMA などの細胞骨格蛋白をさまざまな割合で共発現すること、また、一部の GFAP 発現細胞は間葉系幹細胞マーカーであるネスチン、A3 蛋白質や Thy-1 を発現することを明らかにした。さらに、筋線維芽細胞は、線維化の進行に伴い増殖活性を示す一方で、アポトーシスで消退することで瘢痕巣を形成することが分かった。肝硬変での筋線維芽細胞は、肝星細胞や間葉系幹細胞から持続的に誘導され、かつ増殖と消退を繰り返すことで増悪病態である偽小葉の形成に係わることを明らかにした。

肝硬変においては、肝細胞と胆管上皮の双方に分化し得る肝前駆細胞（oval 細胞）の異常な出現がみられる。そこで、第 3 章では、TAA 誘発肝硬変モデルを用いて、肝前駆細胞の特性を解析している。肝前駆細胞は、線維性架橋に出現し、GFAP と CK19（胆管上皮マーカー）

を共発現することが分かった。肝前駆細胞から生じた異常な胆管（偽胆管）は上皮－間葉転換（EMT）を介して筋線維芽細胞を誘導するとされるが、偽胆管上皮にはSMA発現はみられなかった。一方、孤在性の肝前駆細胞には、癌肝細胞マーカーであるAFPやβカテニンの発現がみられ、さらに、マイクロダイゼクション法による架橋部位の解析により肝発癌に関わるWnt2、Wnt4やグリピカン-3のmRNA発現の増加が示された。肝硬変でみられるGFAP発現細胞は、第2章で示した筋線維芽細胞の誘導のみならず、肝硬変の終末病態である肝発癌にも関わる可能性が示された。

以上の研究成果は、肝線維化/肝硬変の病理発生には、GFAPを発現する筋線維芽細胞や肝前駆細胞が重要な役割を演じること、さらに、化学物質の肝毒性や肝発癌試験における新たな病理学的評価手法としてGFAP発現細胞を指標にした解析が有用であることを提示している。本研究は、肝疾患の病理発生機序の解明に向けた医学・獣医学における基礎・応用研究のさらなる発展に資するものであり、従って、最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。