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学位授与の日付	平成24年3月31日	
論文名	Pathological Studies on the Biliary Fibrosis with Special Reference to Macrophage Properties in Relation to Myofibroblast Development (胆管線維症に関する病理学的研究：特に筋線維芽細胞形成との関連におけるマクロファージの特性について)	
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論文要旨

Introduction

There is growing impact of cirrhosis owing to public health burden associated with significant morbidity and mortality worldwide. Parenchymal injury induced by ischemia, viruses, chemicals and malnutrition is the key initial starting point of hepatic fibrosis. Injured hepatocytes secrete cytokines, which can recruit inflammatory cells into damaged sites. Infiltrating cells, in turn, orchestrate a series of remodeling events that culminate in reparative fibrosis. However, hepatic fibrosis can progress towards cirrhosis, an end stage condition, depending on degrees of injury or type of injurious agents. Biliary fibrosis, a subtype of hepatic fibrosis, is developed due to cholangiocyte injury such as enteric bacterial infection and autoimmunity. The pathogenic mechanism of biliary fibrosis is poorly understood. Animal models for biliary fibrosis should represent a series of events of fibrogenesis in the Glisson's sheath, and may serve as a useful tool to clarify the underlying mechanisms, as well as to assess efficacy of anti-fibrotic agents.

Basically, fibrosis is evoked through various functions of reactive cells including inflammatory cells, vascular cells, and mesenchymal cells. Out of them, macrophages, which are divided into exudate macrophages, resident macrophages and dendritic cells, have been implicated to play pivotal roles in hepatic fibrosis. Macrophages are heterogeneous in nature, and their functions easily change

depending on microenvironmental conditions. Soluble factors secreted by macrophages are capable of inducing myofibroblast development. The myofibroblasts are so-called contractile cells expressing α -smooth muscle actin (α -SMA), and contribute to excessive deposition of collagens. Fibrotic lesions may be developed via interactions between macrophages and myofibroblasts. In hepatic fibrosis, hepatic stellate cells (HSCs) are considered to be the principal progenitor of myofibroblasts; in addition to the origin, however, cellular properties of myofibroblasts in biliary fibrosis remain to be determined. Understanding the properties of macrophages and myofibroblasts in biliary fibrosis may provide a new avenue in exploring agents for cell type specific therapeutic interventions.

A series of studies were conducted in order to investigate the properties of macrophages in relation to myofibroblast development in biliary fibrosis. In Chapter 1, immunophenotypical analyses were performed to clarify the distribution of hepatic macrophages in developing rat livers. To understand the appearance of macrophages in spontaneous lesions, in Chapter 2, macrophages and myofibroblasts in *Fasciola*-infected cattle liver with biliary cirrhosis were characterized with immunohistochemistry. Based on results in Chapters 1 and 2, to more clarify the pathogenesis of biliary fibrosis, the author established the acute (Chapter 3) and chronic (Chapter 4) animal models for biliary fibrosis induced in rats by α -naphthylisothiocyanate (ANIT; hepatotoxicant causing cholangiocyte injury); detailed immunophenotypical characteristics of macrophages and myofibroblasts were analyzed to shed some light behind the pathogenesis. Finally, in Chapter 5, the author compared in macrophage and myofibroblast properties between acute and chronic ANIT-induced biliary fibrosis.

Chapter 1: Hepatic Macrophages in Developing Rat Livers

Little is known about hepatic macrophages in fetuses and neonates of rats. To establish baseline data, liver samples of F344 rats obtained on gestational days 18-20, neonate days 1-21, and adult weeks 5-35 were analyzed immunohistochemically (n=2-5/examination point). Macrophages positive for CD68 (ED1; implying phagocytic activity) were mostly predominant at prenatal period, suggestive of ingestion of apoptotic cells; macrophages positive for CD163 (ED2; relating to production of proinflammatory factors), CD204 (SRA-E5; relating to lipid metabolism) and major histocompatibility complex (MHC) class II (MHC II) (OX6) appeared mainly in postnatal life. CD163+ and CD204+ cells were present along the sinusoids, indicating Kupffer cells; cells expressing MHC II were limited in the Glisson's sheath, indicative of interstitial dendritic cells. In developing livers, macrophages can express heterogeneous immunophenotypes, suggesting that macrophages with divergent properties participate in liver development and structure formation.

Chapter 2: Properties of Macrophages and Myofibroblasts in *Fasciola*-infected Cattle Biliary Cirrhosis

To understand what types of macrophages can take part in spontaneous biliary fibrogenesis, *Fasciola*-infected cattle livers (n=8) with biliary cirrhosis were analyzed using antibodies to CD68, CD163 (AM-3K), CD204 (SRA-E5) and Iba-1 (a protein involved in calcium signaling, phagocytosis and chemotaxis) for macrophages, and to α -SMA, vimentin and desmin for myofibroblasts. CD68+ and CD163+ cells were significantly increased in cirrhotic lesions. Myofibroblasts expressed α -SMA, vimentin and desmin in varying degrees; the desmin expressing cells were relatively fewer in number. These findings indicated that CD68+ and CD163+ macrophages could take part directly in biliary fibrogenesis, and that myofibroblasts expressed various cytoskeletons, mainly vimentin and α -SMA.

Chapter 3: Immunohistochemical Analyses of ANIT-induced Acute Cholangiocyte Injury Lesions in rats

Section I: Immunophenotypes of Macrophages and Myofibroblasts

To establish an animal model for biliary fibrosis, cholangiocyte injury in the Glisson's sheath was induced in F344 rats by a single intraperitoneal injection of ANIT (75 mg/kg). Liver samples were obtained on post injection (PI) days 0 (control), 1, 2, 3, 5, 7, 9 and 12 (n=3/examination point). Macrophages reacting to MHC II consistently increased during the entire observation period. CD68+ and CD204+ cells appeared later than MHC II+ cells from PI day 2 with significant increase; these macrophages decreased gradually at mid and late stages. CD163+ cells increased transiently on PI day 3. There were double positive cells to MHC II/CD68 or MHC II/CD163. Vimentin+ and desmin+ myofibroblasts appeared at early and mid stages, and α -SMA+ myofibroblasts were developed at mid and late stages. Myofibroblasts co-expressing both vimentin and α -SMA were greater in number than those expressing both desmin and α -SMA. Monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor- β 1 (TGF- β 1) mRNA increments appeared to be related to macrophage infiltration at early stages and myofibroblastic differentiation at mid and late stages, respectively. Depending on progression stages (cholangiocyte injury, biliary fibrosis and healing), macrophages and myofibroblasts display various immunophenotypes; particularly, it was found that MHC II+ cells might have central roles, and that α -SMA+ myofibroblasts could be formed in mid and late stages.

Section II: Regenerative Potential of Biliary Fibrosis-involving Cells

By using immunohistochemistry with antibodies to nestin (an intermediate filament protein used as a marker for progenitor of nerve cells) and Ki-67 (a marker of proliferating cells), regenerative potential was investigated in injured cells of the Glisson's sheath with biliary fibrosis. Nestin expression was seen in cholangiocytes, myofibroblasts and endothelial cells; some of these cells reacted to Ki-67. After injury, cells of these types might have potential to regenerate as their possible progenitors. Nestin would be used as an indicator for regeneration in hepatotoxicity.

Chapter 4: Immunophenotypes of Macrophages and Myofibroblasts in ANIT-induced Chronic Biliary Fibrosis in rats

By repeated injections of ANIT (30 mg/kg for 5 weeks followed by 75 mg/kg until 19 weeks, intraperitoneally, once weekly) for 19 weeks, slowly progressive biliary fibrosis model, resembling human counterpart lesions, was established in F344 rats. Liver samples were examined at post-first injection (PFI) weeks 3, 7, 10, 13, 16 and 19 (n=4/examination point). MHC II⁺ macrophages significantly increased consistently during the entire observation. CD68⁺ and CD204⁺ macrophages showed a significantly increased number from PFI weeks 7 to 19, being later than MHC II⁺ cells. CD163⁺ macrophages significantly increased only at late stages (PFI weeks 13-19). Interestingly, there were double positive cells to MHC II/CD68 or MHC II/CD163, and the distribution of MHC II⁺ cells and CD204⁺ cells was limited in the affected Glisson's sheath and the sinusoids of hepatic lobules, respectively. Vimentin⁺ and α -SMA⁺ myofibroblasts appeared at mid and late stages, corresponding with increased TGF- β 1 mRNA; however, double positive cells to vimentin and α -SMA were few, indicating that there was difference in nature between them. Desmin expression was very faint. This study showed that macrophages exhibited heterogeneous properties depending on stages and locations; in particular, MHC II⁺ cells might play crucial roles in biliary fibrosis. In association with such macrophage appearance, myofibroblasts expressing cytoskeletons (particularly, vimentin and α -SMA at mid/late stages) might participate in biliary fibrosis.

Chapter 5: Comparisons of Macrophage and Myofibroblast Properties between Acute and Chronic Rat Biliary Fibrosis Models

The properties of macrophages and myofibroblasts were compared between ANIT-induced acute and chronic biliary fibrosis models. In both models, MHC II⁺ macrophages consistently increased throughout the entire experiment period, followed by increments of CD204⁺ and CD68⁺ macrophages. CD163⁺ macrophages increased transiently in acute ANIT model and at the late stages in chronic ANIT model. These findings indicate that MHC II⁺ macrophages, which may be stimulated by cholangiocytes injured by ANIT, can induce macrophages reacting to CD204 and CD68, and participation of CD163⁺ macrophages is slight in acute ANIT model and delay in chronic ANIT model. Vimentin⁺ and desmin⁺ myofibroblasts increased at early and mid stages in acute ANIT model, whereas vimentin expression was almost consistent and desmin expression was faint in chronic ANIT model; α -SMA was expressed in myofibroblasts at mid and late stages in both models. Vimentin⁺ portal fibroblasts and desmin⁺ HSCs in acute ANIT model, and vimentin⁺ portal fibroblasts in chronic ANIT model may be regarded as precursors of α -SMA⁺ myofibroblasts. As compared with macrophage properties (exclusively CD68⁺ and CD163⁺ macrophages) in perivenular fibrosis model previously reported, it was found that antigen presenting cells (MHC II⁺ macrophages) in the Glisson's sheath can regulate the progressive biliary fibrosis.

Conclusions

1. Heterogeneous macrophage populations participate in rat liver formation; they show different distributions and functions, depending on age.
2. In *Fasciola*-infected cattle livers with cirrhosis as spontaneous lesions, CD68+ (phagocytosis) and CD163+ (proinflammatory factor production) macrophages are more predominant in fibrotic lesions; myofibroblasts variously express α -SMA and vimentin.
3. In ANIT-induced acute and chronic biliary fibrosis, macrophages exhibit heterogeneous properties (phagocytosis, proinflammatory factor production, lipid metabolism, and MHC II expression), demonstrable with immunohistochemistry, depending on stages of lesion progress in the damaged Glisson's sheath. Of them, MHC II+ macrophages may have central roles in the lesion development as the first responder.
4. Additionally, myofibroblasts in ANIT-induced lesions in the Glisson's sheath display various cytoskeletons such as vimentin, desmin and α -SMA; in biliary fibrosis, desmin (although the reactivity is faint) tended to be expressed at early stages, and vimentin and α -SMA at mid and late stages.
5. Nestin expression would be used as an indicator for regeneration in injured cells in the Glisson's sheath.
6. Because ANIT-induced lesions in the Glisson's sheath resemble human biliary fibrosis, these lesions would be beneficial to clarify the underlying mechanisms of biliary fibrosis, as well as to test efficacy of anti-fibrotic agents as animal models.

審査結果の要旨

ウイルス感染、中毒、虚血などによる肝細胞傷害後に生じる線維化は、肝硬変へと進展し、重度の健康障害を招く。胆管傷害後の線維化も進行すれば肝硬変に至る。肝硬変は慢性的に生じる線維化で、その形成には、反応するマクロファージとコラーゲンなどの細胞外基質を産生する筋線維芽細胞が中心的な役割を担う。しかし、線維化の病理発生機序の全貌は不明な点が多い。マクロファージは貪食活性、炎症性因子の産生、酸化ストレス因子の産生、脂質代謝、抗原提示能など多彩な特性を現す細胞群であり、筋線維芽細胞は α -平滑筋アクチン (α -SMA) などの細胞骨格を種々の程度で発現する拘縮性細胞で、常在する線維芽細胞に由来する。しかし、胆管線維化に出現するマクロファージの機能的性状や筋線維芽細胞の細胞特性は未だ明らかにされていない。これら細胞の基本的な性状を解明し、相互の関連性を追究することは、肝硬変を治療する方策に繋がる。本研究では、ラットの肝発生過程におけるマクロファージの特性と、胆管上皮を特異的に傷害する α -naphthylisothiocyanate (ANIT) をラットに投与することで作出した

急性と慢性の胆管線維化モデルを用いて傷害されたグリソン鞘に出現するマクロファージと筋線維芽細胞の特性を、主として免疫組織化学的に解析している。得られた成績の概要は以下の通りである。

第1章では、発生過程（胎子、新生子、成体）の肝における正常なマクロファージの特性を解析している。その結果、胎子では貪食活性の高いCD68発現マクロファージが、新生子から成体では抗原提示（MHCクラスII発現）マクロファージが主に出現すること、また肝常在マクロファージであるCD163発現クッパー細胞は生後に現れ始め、肝組織構築に係わるとともに脂質活性と関連するCD204を発現することが分かった。さらに、MHCII発現マクロファージはグリソン鞘に、CD163とCD204発現マクロファージは類洞に沿って出現することを示した。肝発生過程で出現するマクロファージ機能は発生時期に依存して異なること、そして部位特異的なマクロファージが存在することを明らかにした。

第2章では、ウシの肝蛭感染による胆管線維症の病態を解析し、線維化部位ではCD68とCD163発現マクロファージが増加し、それに伴いビメンチンと α -SMA陽性の筋線維芽細胞が形成されることを示した。寄生虫感染による胆管線維化に出現するマクロファージと筋線維芽細胞の特性を初めて明らかにした。

第3章では、ANITの単回投与による胆管傷害後の急性の線維化モデルを作出し、12日間に亘り病態の推移を解析している。その結果、MHCII発現マクロファージは初期から後期まで常に増加し、それに続いてCD68とCD204発現マクロファージがやや遅れて増し始めることを示した。なお、CD163発現マクロファージは一過性に増加したのみであった。筋線維芽細胞は、前半はビメンチンとデスミンを、後半からは α -SMAを発現することが示された。さらに、MCP-1はマクロファージの出現と、TGF- β 1は α -SMA陽性の筋線維芽細胞の形成と関連することを明らかにした。また、傷害後に再生する胆管上皮と筋線維芽細胞などの間質細胞がネスチンを発現することを示した。

第4章では、ANITを19週間に亘って投与し、形成されるグリソン鞘の線維化病変を解析している。その結果、MHCII発現マクロファージは観察期間を通じ常に高頻度で出現し、CD68とCD204発現マクロファージがそれに続いて増加し、CD163発現マクロファージは後期のみ出現することが示された。また、デスミン陽性の筋線維芽細胞の出現は少なく、ビメンチンと α -SMA陽性の筋線維芽細胞が線維化病変の形成に深く係わることを示した。

第5章では、ANIT誘発の急性と慢性の胆管線維化病変とすでに報告されている肝細胞傷害後の実質線維化に出現するマクロファージの特性を比較し、ANIT誘発の胆管線維化ではMHCII発現マクロファージが中心的な役割を演じるのに対し、実質線維化ではCD68とCD163発現マクロファージが主体となることを示した。また、 α -SMA陽性の筋線維芽細胞が胆管線維化の進行に深く係わることを明らかにした。

以上の研究により、胆管線維化過程において、病変形成に伴い異なる機能のマクロファージが出現すること、さらに α -SMA 発現の筋線維芽細胞が線維化の進行において重要な役割を演じることが明らかとなった。本研究は、肝硬変、特に胆管線維化に起因する肝硬変の病理発生機序を解明する上で極めて重要な知見を提示しており、医学・獣医学の基礎・応用研究の更なる発展に資するものである。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。