称号及び氏名	博士（獣医学）	Rajapaksa Rathnayaka Mudiyanselage Kavindra Kumara Wijesundera
学位授与の日付	平成２６年９月３０日	
論　文　名	Pathogenesis of Thioacetamide-Induced Rat Hepatic Fibrosis Based on Heterogeneous Functions of Macrophages （マクロファージの多様な機能特性に基づいたチオアセトアミド誘発ラット肝線維化の病理発生）	
論文審査委員	主査　山手　丈至 副査　松尾　三郎 副査　中村　洋一	

論文要旨

Introduction

The complications of liver diseases impose a major health burden on human society. Liver cirrhosis, the advanced lesion of repeated fibrosis, represents a common clinical endpoint of all chronic liver diseases and characterized by degenerative/regenerating hepatocytes, diffuse fibrosis and pseudolobules. The advanced fibrosis is at high risk of non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC). The activation of macrophages (Kupffer cells), recruitment of inflammatory cells, and subsequent release of cytokines by them are responsible for liver lesion development in the lineage of hepatocyte injury, fibrosis and cirrhosis. The pathogenesis of liver fibrosis and cirrhosis is not yet clearly defined. It is important to investigate the pathogenesis of such lesions, which will open new opportunities for therapeutic targets.

It is known that macrophages play roles in the pathogenesis of liver injury and subsequent fibrosis. In pathological lesions, macrophages are classified as classically activated (M1) and alternatively activated (M2) macrophages. M1-macrophages are induced by IFN-γ and function mainly in inflammatory

reactions with tissue destruction, while M2-macrophages are activated by IL-4 and resolve inflammatory responses which lead to fibrosis and tissue remodeling (healing). However, M1-/M2-macrophage polarization is not yet defined in toxin-induced liver lesions.

A series of studies were conducted to investigate the M1-/M2-macrophage properties in thioacetamide (TAA)-induced acute liver injury and cirrhosis. In Chapter 1, to establish the paradigm of M1-/M2-macrophage polarization, the author immunophenotypically analyzed macrophage properties after a single injection of TAA. Additionally, the author investigated the conditions of macrophage depletion in the TAA-induced acute liver lesion. In Chapter 2, the author analyzed immunophenotypes of macrophages in TAA-induced rat liver cirrhosis, focusing on macrophage properties and cytokine profiles in three different pathological conditions: developing fibrous bridges (FBs)/pseudolobules (PLs), Glutathione S-transferase placental form (GST-P)-positive/-negative PLs, and adipophilin (Adp)-positive/-negative PLs. In Chapter 3, finally, comparison of M1-/M2-macrophage polarization properties was made between TAA-induced acute liver injury and cirrhosis.

Chapter 1: Heterogeneity of macrophages appearing in thioacetamide (TAA)-induced acute rat liver injury and fibrosis

Section I: M1-/M2-macrophage polarization in TAA-induced acute rat liver injury and fibrosis

To investigate the M1-/M2-macrophage polarization, rats were injected with TAA, once. Liver samples were collected on post-injection (PI) hour 10, and days 1, 2, 3, 5, 7, and 10. Coagulative necrosis of hepatocytes was observed in centrilobular areas on PI day 1, reaching the maximum on PI day 2. Fibrosis began to be observed on PI day 3. On hour 10, M1-related factors and M2-related factors (IL-4) were already increased, followed by increased expressions of IL-10 and TGF-β1 for M2 on PI days 1 to 3 when centrilobular lesions and subsequent reparative fibrosis occurred. On hour 10, $CD204^+$ and MHC class II^+ macrophages already increased in the intact periportal and Glisson's sheath regions. On days 1-3, CD68 (for M1) and CD163 (for M2) macrophages were increased, and they showed double-positive to CD68/MHC class II and CD163/CD204, indicating that MHC class II^+ and $CD204^+$ macrophages had M1- and M2-polarization, respectively. The M1-/M2-macrophage paradigm is useful to analyze the acute hepatotoxicity.

Section II: Characterization of Iba1- and Galectin-3 (Gal-3)-positive macrophages based on macrophage polarization in TAA-induced acute rat liver injury

and fibrosis

To evaluate the M1-/M2-macrophage properties of $Iba1^+$ and $Gal\text{-}3^+$ macrophages, centrilobular lesions were induced in rats using the same method. Macrophages reacting to Iba1 and Gal-3 were increased in numbers on PI days 1 to 3, peaking on PI day 2. IFN-γ and MCP-1 for M1 were increased on PI day 1, whereas IL-10 and TGF-β1 for M2 were increased from PI days 1 to 3. $Iba1^+$ and $Gal\text{-}3^+$ macrophages reacted to $CD68^+$ and $CD163^+$, respectively. $Iba1^+$ and $Gal\text{-}3^+$ macrophages had the M1- and M2-polarization, respectively. $Iba1^+$ and $Gal\text{-}3^+$ macrophages also participated in the pathogenesis of the acute liver injury and fibrosis.

Section III: Characterization of M1-/M2-macrophage functions in TAA-induced acute liver injury by macrophage depletion with clodronate

Macrophages were depleted in TAA-induced acute liver lesions by clodronate (CLD). Liver samples were collected on PI days 1, 2, and 7. In CLD-treated rats, mainly on PI days 1 and 2, all macrophage populations reacting to CD68, CD163, Iba1, MHC class II, and Gal-3 were significantly decreased; the hepatocyte lesion was less severe. However, dystrophic calcification occurred in injured areas. Depletion of macrophages resulted in reduced tissue injury at the early stages and difficulty in removal of cell debris at the late stages.

Chapter 2: Characterization of macrophages in cirrhosis induced by repeated injections of thioacetamide (TAA)

Section I: Histopathology of cirrhosis induced by repeated injections of TAA

To establish an animal model for cirrhosis, rats were repeatedly injected with TAA. Liver samples were collected at post-first injection (PFI) weeks 5, 10, 15, 20, 25, and 32. At PFI weeks 5 and 10, hepatocyte damage progressed gradually. At PFI week 15, collagenous fibrous bridges (FBs) were formed, separating liver parenchyma into pseudolobules (PLs). Thereafter, these lesions were exacerbated with time. TAA-induced rat liver cirrhosis resembles human micro-nodular cirrhosis.

Section II: M1-/M2-macrophage polarization in cirrhosis induced by repeated injections of TAA

In the above cirrhosis model, macrophage immunophenotypes were investigated by separating the FBs and PLs. Myofibroblasts were most pronounced in FBs at PFI week 15. $CD68^+$, $CD163^+$, $CD204^+$, $Iba1^+$ and $Gal\text{-}3^+$ macrophages appeared in both FBs and PLs, peaking at PFI week 15, with greater number in FBs; the appearance corresponded to increased levels of both M1 and M2 factors. MHC class II^+ macrophages were increased in FBs at early

stages, but the increased number was gradually decreased onwards. The double-positive cells to CD68/CD163 showed 100% co-localization at PFI weeks 10 and 15. Double immunolabeling for CD68 and CD163 in Iba1$^+$ or Gal-3$^+$ macrophages showed similar percentage. Macrophages with different functions participated in cirrhosis, particularly in the FB formation, without clear discrimination of M1-/M2-polarization.

Section III: Appearance of macrophages in glutathione S-transferase placental form (GST-P)-positive and -negative pseudolobules (PLs) in TAA-induced cirrhosis

Macrophage properties were analyzed between GST-P-positive and -negative PLs in the cirrhosis model. GST-P-positive foci were clearly observed at PFI week 15. GST-P-positive and -negative PLs were distinguishable at PFI weeks 20-32. DNA microarray revealed the upregulation of pre-neoplastic genes in GST-P-positive PLs at PFI week 32. CD68$^+$, CD163$^+$, CD204$^+$, Gal-3$^+$ and Iba1$^+$ macrophages were greater in numbers in GST-P-positive PLs, whereas MHC class II$^+$ macrophage number was more predominant in GST-P-negative PLs. Expressions for both M1 and M2 factors were higher in GST-P-positive PLs. There was no clear difference in the M1-/M2-macrophage polarization in GST-P-positive lesions. Macrophages except for MHC class II$^+$ macrophages might contribute to the development of hepatic pre-neoplastic lesions through both M1- and M2-related factors.

Section IV: Macrophage properties in adipophilin (Adp)-positive and -negative pseudolobules (PLs) in TAA-induced cirrhosis

The relationship between macrophages and Adp-positive PLs was investigated in the cirrhosis model. Gradually increased Adp expression was seen from PFI week 5. Adp-positive and -negative PLs were distinguishable at PFI week 20 onwards. DNA microarray at PFI week 32 revealed the upregulated steatogenic genes in Adp-positve PLs. CD68$^+$, CD163$^+$, CD204$^+$, Iba1$^+$, and Gal-3$^+$ macrophages were greater in Adp-positive than -negative PLs. M1 and M2 factors were both higher in Adp-positive PLs. There was no clear difference in the M1-/M2-macrophage polarization in Adp-positive lesions. On the contrary, MHC class II$^+$ macrophages were increased at the early stages at weeks 5 and 10. Macrophages with heterogeneous functions participated in steatosis in cirrhosis through M1- and M2-related factors.

Chapter 3: Comparison in macrophage properties between thioacetamide

(TAA)-induced acute liver injury/fibrosis and cirrhosis in rats

In TAA-induced acute liver injury, macrophages with different immunophenotypes showed a transient increase, while in TAA-induced cirrhosis, these macrophages relatively underwent a constant increase.

In TAA-induced acute liver injury, there were distinguishable injurious stages governed by M1 factors, followed by remodeling/fibrotic stages with M2-predominance; on the contrary, in the TAA-induced cirrhosis, both M1-/M2-macrophage polarization occurred contemporarily.

In both TAA-induced acute liver injury and cirrhosis, MHC class II^+ macrophages became activated early, which could induce other types of macrophages.

In acute liver injury and fibrosis, $Iba1^+$ and $Gal\text{-}3^+$ macrophages polarized into M1- and M2-macrophages, respectively. In TAA-induced cirrhosis, $Iba1^+$ and $Gal\text{-}3^+$ macrophages showed M1- and M2-polarization, simultaneously.

Conclusions

Based on M1-/M2-macrophage polarization paradigm, macrophage properties were analyzed in TAA-induced acute liver injury and cirrhosis in rats.

1. In both TAA-induced acute liver injury and cirrhosis, macrophages with heterogeneous properties participated in lesions.
2. In acute liver injury, M1-macrophages (mainly $CD68^+$) and M1 factors took part in early lesions (tissue damage), whereas M2-macrophages (mainly $CD163^+$) and M2 factors contributed to fibrosis and subsequent healing.
3. In acute liver lesions, macrophages reacting to MHC class II and Iba1 had M1 type, whereas those reacting to Gal-3 and CD204 showed M2 type.
4. In acute liver injury, MHC class II^+ and $CD204^+$ macrophages appeared in and around Glisson's sheath before lesion commencement, indicative of their initial responders for injury.
5. In acute liver injury, depletion of macrophages by clodronate resulted in less severe hepatocyte injury at early stages, but dystrophic calcification occurred at late stages, presumably due to difficulty in macrophage removal of cell debris.
6. In cirrhosis, macrophages with various functions participated in fibrous bridge, and in GST-P-positive (pre-neoplastic lesion) and Adp-positive (steatosis) pseudolobules through both M1/M2 factors.
7. Clarifying macrophage properties would be beneficial to understand the pathogenesis of hepatic fibrosis/cirrhosis, and could then lead to possible therapeutic strategies for the intractable fibrosis.

審査結果の要旨

進行性の肝線維化はヒトや動物に重大な健康障害をもたらす。その進展した病型である肝硬変は、肝炎ウイルス感染、アルコール中毒、非アルコール性脂肪性肝炎、化学物質による中毒などが原因となり生じる難治性疾患とされている。肝硬変は、組織学的には、肝細胞の変性・壊死・不完全再生による線維性架橋、偽小葉、偽胆管の形成により特徴付けられ、増悪すれば肝癌に至る。肝臓に生じる線維化は、浸潤したマクロファージが産生する因子により誘導される筋線維芽細胞が、コラーゲンなどの細胞外基質を過剰に産生することで形成される。すなわち、肝線維化の進展には、マクロファージが中心的な役割を担うが、その機能特性の全貌は未だ解明されていない。

近年傷害部位に出現するマクロファージは、初期に浸潤する貪食能の高い古典的活性化マクロファージ（M1 型）と、その後の組織線維化に係わる修復性マクロファージ（M2 型）に大別される。この概念は M1/M2 マクロファージ分極化と称される。本研究では、ラットを用いてチオアセトアミド(TAA)誘発の急性肝傷害と、TAA 連回投与による肝硬変におけるマクロファージの特性を M1/M2 分極化の観点から解析している。得られた一連の結果の概要は以下のとおりである。

第 1 章では、TAA 単回投与による急性肝傷害におけるマクロファージとその関連因子の解析を試みている。その結果、M1 型誘導に関わる INF-γ、TNF-α、IL-6 と、M2 型誘導に関わる IL-4 の発現が、肝細胞傷害に先立ちすでに増加していること、そしてこれら因子はグリソン鞘既存の MHC クラス II と CD204 発現マクロファージから産生される可能性を示した。この初期反応に続いて、CD68 発現 M1 型と CD163 発現 M2 型が、肝小葉中心部の傷害部位に誘導され、それに一致して M2 型から線維化に係わる TGF-β1 や IL-10 が産生されることが分かった。さらに、傷害部位に出現するマクロファージの機能特性を免疫組織化学的に解析し、M1 型では、CD68 に加えて、MHC クラス II と Iba1 が発現すること、M2 型では、CD163 に加えて、CD204 とガレクチン-3 が発現することを見出した。

また、第 1 章では、クロドロネート前投与によるマクロファージ枯渇条件下での TAA 誘発急性病変を解析している。その結果、初期にはマクロファージが枯渇していることから、傷害因子の産生が低下し、よって肝小葉中心部の凝固壊死の形成が軽減されるが、修復過程においては、マクロファージによる傷害組織の排除がなく、その為に異栄養性石灰沈着が生じ、治癒が遅延することが分かった。肝病変形成におけるマクロファージ機能の二面性（M1 型/M2 型）が明らかとなった。

第 2 章では、TAA を 35 週間週 2 回投与することで誘発された肝硬変の多様な病態をマクロファージの M1/M2 分極化の観点から解析している。このモデルでは 15 週以降に線維性架橋による偽小葉の形成が始まること、その組織学的特性はヒトの小結節性肝硬

変に類似することを示した。偽小葉と線維性架橋との比較、アディポフィリン発現（脂肪性肝炎状態）と非発現の偽小葉との比較、胎盤型グルタチオン S-トランスフェラーゼ(GST-P)発現（前腫瘍性病変）と非発現の偽小葉との比較では、線維性架橋、アディポフィリン陽性偽小葉、GST-P 陽性偽小葉において、それぞれの比較対照に比べ、より多くのマクロファージが出現することを明らかにした。また、それらのマクロファージは M1 型と M2 型の双方の特性を同時に発現し、かつ M1 型と M2 型関連因子も連動して増加していることを明らかにした。肝硬変は、M1 型と M2 型マクロファージが相互に複雑に関連することで形成されることが分かった。

第 3 章では、TAA 投与により誘発された急性肝傷害と慢性病変である肝硬変におけるマクロファージの特性を、より詳細に比較・解析している。その結果、急性病変では、初期の傷害過程とそれに続く線維化の治癒過程では、それぞれ M1/M2 分極化に基づいて病理発生を解析できること、一方、肝硬変では、線維性架橋、アディポフィリン陽性偽小葉、GST-P 陽性偽小葉において、M1 型と M2 型マクロファージとそれぞれの関連因子が同時に増加し複雑に関連することで肝硬変の増悪病態である線維化、脂肪化、腫瘍化が誘導されることが分かった。

以上の研究成果は、肝線維化と進行性の肝硬変の病理発生には、M1 型と M2 型マクロファージが連続的あるいは相互的に関与すること、さらに、化学物質による肝毒性の新たな評価手法として M1/M2 分極化による機能特性の解析が有用であることを示している。本研究は、毒性病理学的な観点から、医学・獣医学の基礎・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。