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論文名	Pathological study on liver homeostasis and hepatotoxicity based on macrophage functions and autophagy in rats (マクロファージ機能とオートファジーに基づいたラットの肝恒常性と肝毒性に関する病理学的研究)	
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論文要旨

Introduction

Liver is a crucial organ in the body, consisting of parenchymal hepatocytes and non-parenchymal cells (Kupffer cells, hepatic stellate cells (HSCs), cholangiocytes and endothelial cells). The relationship between these parenchymal and non-parenchymal hepatic cells is important for the maintenance of liver functions and homeostasis. Hepatic macrophages are considered a key player for initiating and shaping of immune responses in liver through phagocytosis, cytokine production and antigen presentation. Hepatic macrophages mainly include Kupffer cells; however, liver harbours hepatic macrophages with different immunophenotypes such as exudative macrophages and antigen presenting cells. In pathological condition, hepatic macrophages appear as M1 macrophages (classically activated macrophages) and M2 macrophages (alternatively activated macrophages) based on the type 1 or 2 helper T-cell polarization. Interestingly, depending on microenvironmental conditions, M1 macrophages could shift into M2 phenotypes or vice versa. Therefore, it is interesting to investigate which type of macrophages may contribute to physiological and pathophysiological adaptations including hepatotoxicity. Additionally, liver has a basal level of autophagy, which is an evolutionarily conserved and strictly regulated lysosomal pathway for intracellular degradation. Autophagy is characterized by the formation of

double-membrane vesicle termed autophagosome, demonstrable with LC3B (microtubule-associated protein 1 light chain 3 beta, an essential component of autophagosome) immunohistochemistry. Autophagy contributes to hepatic homeostasis and is also activated in a variety of liver diseases including hepatotoxicity.

To investigate the functional properties of hepatic macrophages and autophagy in liver homeostasis and hepatotoxicity, a series of studies were carried out. These functional properties were mainly histopathologically analyzed using rats under low dose of lipopolysaccharide (LPS) or clodronate (CLD) treatment. The obtained data in these studies would provide important information on heterogeneous macrophage functions and autophagy participating in liver homeostasis and pathology.

Chapter 1: Characterization of rat hepatic macrophages participating in liver homeostasis

Section I: Effects of empty liposomes on hepatic macrophage populations in rats

To identify the transient effects of empty liposomes on hepatic macrophages, F344 rats were injected with liposome (10 ml/kg BW, iv). Serum and liver samples were collected one day after injection. The numbers of hepatic macrophages reacting to CD163, CD68 and MHC class II were significantly increased in liposome-treated rats. Although there were no changes in liver histoarchitecture, hepatocytes showed increased proliferating activity with increased gene profiles related to cell cycle. Interestingly, AST and ALT levels were significantly decreased, and mRNA expression of MCP-1, IL-1 β and TGF- β 1 were significantly increased. It was found that hepatic macrophages activated by liposomes influence liver homeostasis.

Section II: Immunophenotypes of depleting and repopulating hepatic macrophages in rats treated with CLD

To characterize the depleting and repopulating hepatic macrophages, F344 rats were injected with liposomal CLD (50 mg/kg BW, iv). Serum and liver samples were collected on post-injection (PI) 1, 3, 5, 7, 9 and 12 days. Hepatic macrophages detectable by different antibodies were decreased in varying degrees after CLD injection. CD163⁺ Kupffer cells were the most susceptible to CLD, which were almost completely depleted on PI days 1-12. CD68⁺ macrophages were transiently reduced on PI day 1 and then, recovered gradually until PI day 12. MHC class II⁺ macrophages were moderately decreased during the observation periods. Histologically, no marked changes were seen in the liver, excluding the increased proliferating activity of hepatocytes on PI day 1. AST, ALP, ALT, total bilirubin and γ -GTP levels were significantly elevated and factors for macrophage induction/activation (MCP-1, CSF-1, IL-6 and IL-4) were increased transiently exclusively on PI day 1, whereas anti-inflammatory factors (IL-10 and TGF- β 1) remain significantly decreased under hepatic macrophage depletion. These findings showed the importance of hepatic

macrophages in liver homeostasis.

Chapter 2: Characterization of hepatic macrophages and autophagy under LPS treatment in rats

Section I: Effects of low dose of LPS on hepatic macrophages and regulatory inflammatory factors in CLD-treated rats

To clarify pathophysiological effects of low dose of LPS on hepatic macrophages and regulatory inflammatory factors, F344 rats were pretreated with CLD (50 mg/kg BW, iv) (CLD+LPS rats) or vehicle liposome (Lipo) (Lipo+LPS rats) 24h before LPS injection (0.1 mg/kg BW, ip) or saline. Serum and liver were collected on LPS PI 0, 6, 12, 24, 48 and 72h. LPS injection did not alter histoarchitectures of the liver, and changes of serum hepatic enzymes were not seen. Macrophages reacting to CD163, CD68 and MHC class II were stimulated to LPS in Lipo+LPS rats and effects on repopulating Kupffer cells were not seen in CLD+LPS rats. Inflammatory-related factors (IL-1 β , IL-6, TNF- α and MCP-1) were dramatically increased at 6h after LPS exposure in Lipo+LPS rats, which was attenuated by macrophage depletion in CLD+LPS rats. On the contrary, anti-inflammatory factors (IL-4 and CSF-1) were significantly elevated in CLD+LPS rats, but did not change in Lipo+LPS rats. Activation of hepatic macrophages by low level of LPS could provide a significant insight into the development of macrophage-based hepatotoxicity

Section II: Characterization of cell specific autophagy in liver and hepatic macrophages under treatment with low dose of LPS in CLD-treated rats

Using the above model, LPS-induced autophagy in hepatocytes and non-parenchymal cells were analysed. Localization of LC3B (autophagy marker) was seen at basal level in hepatocytes, Kupffer cells, HSCs and cholangiocytes in normal rat livers. LPS administration resulted in a markedly increased LC3B expression in hepatocytes of Lipo+LPS and CLD+LPS rats at PI 6h and 12h. In comparisons between these groups, LC3B-positive hepatocytes were markedly lower in CLD+LPS rats. However, LC3B reactivity was increased in GFAP-expressing HSCs in both groups after LPS treatment. LPS injection also induced autophagy in CD163⁺ Kupffer cells, as well as CD68⁺ and MHC class II⁺ macrophages in Lipo+LPS rats. At mRNA level, LC3B and TLR-2 were significantly increased at PI 6h or 12h in Lipo+LPS rats, while these factors was significantly lower in CLD+LPS rats. Low dose of LPS could be used as a potent autophagy inducer in rat livers.

Chapter 3: Roles of hepatic macrophages and autophagy in thioacetamine (TAA)-induced hepatotoxicity in LPS-pretreated rats

Section I: Time dependent protection of LPS in TAA-induced acute liver injury in

rats

To investigate the effects of low dose of LPS in TAA-induced hepatotoxicity as a protective phenomenon, F344 rats were pretreated with LPS (0.1 mg/kg BW, ip) at 2, 6, 12 and 24h before TAA injection (100 mg/kg BW, ip). Serum and liver were collected one day after TAA injection. Histopathologically, TAA injection produced centrilobular necrosis in liver. LPS-pretreated TAA-injected rats at 2h and 6h showed little effect on the hepatic lesions, but the rats at 12h and 24h showed a markedly reduced hepatic lesions. AST, ALP, ALT and total bilirubin values tended to be decreased in LPS-pretreated TAA-injected rats at 12h or 24h. LPS pretreatment showed hepatoprotection against TAA-induced liver lesions depending on time points before TAA injection, particularly at 12h or 24h.

Section II: LPS-mediated cytoprotection in LPS-pretreated rat liver lesions induced by TAA in terms of hepatic macrophages and autophagy

To investigate cytoprotective roles of hepatic macrophages and autophagy in TAA-induced hepatotoxicity under LPS treatment, F344 rats were pretreated with LPS (0.1 mg/kg BW, ip) at 2h and 24h before TAA injection (100 mg/kg BW, ip). Serum and liver were collected on TAA PI 0, 1, 2, and 3 days. Histopathologically, LPS pretreatment at 2h did not reduce the hepatic lesions, whereas the pretreatment at 24h markedly reduced hepatic injury, along with decreased serum hepatic enzymes levels. CD68⁺, CD163⁺ and MHC class II⁺ macrophages were significantly increased after TAA administration, regardless of LPS pretreatment at 2h or 24h. Interestingly, MCP-1, IL-1 β and IL-4 were significantly decreased on day 1 in LPS-pretreated TAA-injured livers at 24h, but were not changed in LPS-pretreated TAA-injured livers at 2h. The autophagy was markedly increased in livers of LPS pretreatment at 2h or 24h. These findings provide information that LPS pretreatment need at least 24h for preparing the microenvironments for cytoprotection against TAA-induced liver injury. Activated macrophages and autophagy may participate in the hepatoprotective mechanism.

Conclusions

Based on the obtained findings, the following conclusions are drawn;

1. Empty liposomes activated hepatic macrophages with different immunophenotypes (mainly, CD68, CD163 and MHC class II), without histopathological changes.
2. CLD injection successfully depleted hepatic macrophages with different immunophenotypes in rat livers. Particularly, CD163⁺ Kupffer cells were the most susceptible to CLD.
3. Low dose of LPS treatment (0.1 mg/kg BW) activated hepatic macrophages without liver lesions. Furthermore, the LPS administration effected liver microenvironments via the increased production of inflammatory mediators.

4. Low dose of LPS treatment induced autophagy in hepatocytes and non-parenchymal cells such as hepatic macrophages and HSCs. Additionally, LPS-activated hepatic macrophages influenced autophagy functions in liver.
5. LPS pretreatment protected development of TAA-induced acute liver lesions depending on time points before TAA injection. Particularly, LPS pretreatment at 24h greatly protected TAA-induced hepatotoxicity, whereas LPS pretreatment at 2h did not show such levels.
6. Hepatic macrophages with different immunophenotypes and activated autophagy might participate in the protective phenomenon against TAA-induced hepatotoxicity.
7. Therefore, the present findings would provide useful information on the properties of hepatic macrophages and autophagy in liver homeostasis and hepatotoxicity, leading to possible insights underlying effective therapeutic strategies against liver lesions.

審査結果の要旨

肝臓は、栄養代謝に加え、医薬品などの化学物質の解毒を行う重要な器官である。化学物質による肝毒性は、主に、化学物質の肝細胞に対する直接的な細胞傷害作用や、肝代謝酵素により生じる化学物質の活性型代謝物に起因する肝毒性発現機序に基づき、その評価が行われている。しかし、肝臓には、実質細胞である肝細胞の他に、非実質細胞としてのクッパー細胞、肝星細胞、血管内皮細胞や胆管上皮細胞などがあり、特に、クッパー細胞などのマクロファージ系の細胞は肝構成細胞の 20~25%を占め、肝恒常性や肝毒性に何らかの影響を与えていると考えられる。しかし、肝マクロファージの機能特性に基づいた肝毒性の発現機序の解析はほとんど行われていない。また、オートファジーは、細胞内老廃物や細胞内侵入微生物を処理する機構として知られているが、肝毒性におけるその役割については未だ十分に研究されていない。このような肝マクロファージやオートファジーは、グラム陰性細菌が産生するリポポリサッカライド (LPS) の影響を受けることが知られている。

そこで、本研究では、ラットを用いて、第 1 章においては、肝マクロファージを活性化あるいは、枯渇させることで肝恒常性にどのような影響が生じるかを解析し、第 2 章では、低用量の LPS が肝マクロファージやオートファジーに及ぼす作用を肝機能との関連で調べている。さらに、第 3 章では、低用量 LPS 前処置における肝毒性物質であるチオアセトアミド (TAA) により誘発される

肝細胞傷害の発現機序を、肝マクロファージとオートファジーとの関連で追究している。この一連の研究に基づいて、肝恒常性や肝毒性に及ぼす肝マクロファージとオートファジーの病態生理学的な役割の重要性を明らかにしている。

第1章では、肝マクロファージに貪食され易いリポソームをラットに投与し、出現する肝マクロファージの免疫組織化学的な特性と肝機能に与える影響を解析している。その結果、リポソーム投与により、CD163 発現 M2 型マクロファージ（主にクッパー細胞）に加え、CD68 と MHC クラス II 発現 M1 型マクロファージが増加・活性化し、さらに AST や ALT などの肝逸脱酵素が低下することが分かった。一方、リポソーム封入クロドロネート投与による CD163 発現 M2 型マクロファージの枯渇状態では、肝細胞傷害が生じていないにも拘わらず AST や ALT 値が上昇することが分かった。すなわち、肝マクロファージの活性化あるいは、枯渇状態により肝機能に変化が生じることを示している。以上より、多様な抗原を発現する肝マクロファージが肝恒常性に係ること、そして肝機能を評価する際には肝マクロファージの状態を把握しておく必要性があることを提示している。

第2章では、肝マクロファージとオートファジー機能に与える LPS の影響を解析している。LPS 投与実験では通常は大量投与が行われ、その際には肝細胞傷害が惹起されることが知られているが、この実験では、腸内細菌叢から産生される微量な LPS が門脈を介して肝臓に流入し肝機能に影響を与えている可能性を想定し、低用量の LPS をラットに投与している。その結果、LPS の低用量（0.1 mg/kg 体重、腹腔内）投与では肝機能障害は生じていないが、CD68 と MHC クラス II 発現 M1 型マクロファージと CD163 発現 M2 型マクロファージが増加し、かつ IL-1 β や TNF- α などの炎症性因子が上昇することが分かった。さらに、LPS 投与により、LC3B 発現のオートファゴソームが、肝細胞のみならず、M2 型マクロファージや肝星細胞においても誘導されることを明らかにした。一方、クロドロネート投与による CD163 発現クッパー細胞枯渇条件下での低用量 LPS の投与では、上記の炎症性因子の産生や肝細胞内のオートファゴソームの誘導が抑制されることが分かった。すなわち、用いた低用量 LPS は肝マクロファージを活性化し、その影響下でオートファジーが誘起されることを示している。

第3章では、ラットを用いて TAA 誘発肝毒性に及ぼす低用量 LPS 投与の影響を追究している。LPS を投与していない TAA 単独投与群では小葉中心性肝細胞凝固壊死が生じ、肝逸脱酵素が上昇していたが、TAA 投与前 2、6、12、24 時間のポイントで LPS を事前投与しておくこと、時間依存性に TAA により惹起される肝機能障害が軽減されることが分かった。その機序としては、第2章で示したように LPS 投与により活性化された肝マクロファージやオートファジーが、TAA 誘発肝細胞傷害の軽減に係ることを示している。特に、CD68 と MHC クラス II 発現 M1 型マクロファージによる傷害組織の残渣処理や、CD163 発現 M2 型マクロファージによる組織修復機能の効果的な作用に係っている可能性を考

察している。

本研究は、肝マクロファージの機能が肝恒常性に深く係っていることを明らかにするとともに、**LPS** はその用量によっては、肝マクロファージやオートファジーを活性化し、肝毒性発現を抑制するとするユニークな知見を見出した。これらの成果は、獣医学や医学、特に病態生理学や毒性病理学分野の基礎研究の新たな展開に資するものと考えられる。よって、本論文の審査ならびに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。