論文要旨

Introduction

　　　　Pathologically, fibrosis develops as a response to injury, which is regarded as a remodeling process. However, continuous injury in liver and kidney culminates in cirrhosis and renal fibrosis, respectively. The pathogenesis of fibrosis is very complicated. Cutaneous wound healing (CWH) is a representative fibrotic lesion.　CWH models in rats and mice have been utilized to clarify a chain of events of fibrogenesis.　Fibrotic lesions consist of various inflammatory cells and deposition of extracellular matrices (ECMs).　Basically, macrophages can regulate fibrogenesis by releasing various fibrogenic factors capable of inducing myofibroblasts. Transforming growth factor-β1 (TGF-β1) is a major fibrogenic factor. Myofibroblasts stimulated by TGF-β1 can produce ECMs such as collagens. Thus, it has been considered that fibrotic lesions may be evoked by interaction between macrophages and myofibroblasts.

　　　　However, macrophages are heterogeneous, being divided into exudative macrophages, resident macrophages and antigen-presenting macrophages/dendritic cells; they show different functions depending on microenvironmental conditions. Although myofibroblasts, a

contractile cell characterized by α-smooth muscle actin (α-SMA) expression, are considered to be induced from pre-existing fibroblasts, the derivation and cellular nature remain to be investigated. Clarifying the relationship and properties of these key cells may lead to therapeutic strategies for the intractable fibrosis.

A series of studies were conducted in order to investigate the detailed characteristics of macrophages and myofibroblasts in cutaneous fibrosis. In the chapter 1, to establish the base-line data, the author immunohistochemically analyzed macrophages in normally-developing rat skin by using different antibodies specific to macrophage subtypes. In the chapter 2, immunophenotypes of macrophages and myofibroblasts appearing in rat CWH model were examined in detail. In the chapter 3, cellular characteristics of macrophages in bleomycin-induced scleroderma (BS) in rats were investigated immunohistochemically in relation to myofibroblast formation. In addition, comparisons of properties of macrophages and myofibroblasts were made between CWH and BS models, to more clarify the divergent features of these cells. Throughout these studies, the author paid attention to expression of galectin-3, because the factor regulates the downstream processes of TGF-β1-mediated myofibroblast activation.

Chapter 1. The kinetics and distribution of different macrophage populations in the developing rat skin

Far less is known about macrophage heterogeneity in normal skin. Rat skin at different anatomical sites (head, dorsal, and abdomen) was obtained on gestational days (GD) 18-20, on neonatal days (ND) 1-21, and at adult weeks (AW) 5-15. Macrophages reacting to ED1 (exudative macrophages with activated phagocytosis), ED2 (resident macrophages producing inflammatory factors), and OX6 (antigen-presenting cells) were assessed in epidermis, dermis or perifollicular areas. There were no differences in positive cell appearance patterns for each antibody among anatomical sites. In epidermis, no positive cells for ED1 and ED2 were seen; in contrast, OX6 cells were seen in GD, and the number increased in ND and AW. In dermis, among macrophages, ED1 cells were the most frequent in GD and ND, whereas ED2 and OX6 cells became more prominent in AW. OX6 cells were predominant in perifollicular areas in late ND and AW. It was demonstrated that heterogeneous macrophages appeared in developing rat skin, based on location and age.

To clarify how different macrophages participate into CWH and BS, in relation with myofibroblast formation, the following studies were conducted.

Chapter 2. Immunohistochemical analyses on macrophages and myofibroblasts appearing in rat CWH

2.1. Histopathology

Excisional punch wounds were made at dorsal skin. Samples were harvested on post-wounding (PW) days 1-26. In CWH, inflammatory phase was noted on PW days 1 to 3,

followed by granulation tissue phase on PW days 5-12; then, tissue remodeling phase was from PW day 15, and the wound almost healed by PW day 26.

2.2. Macrophages and galectin 3-expressing cells

In inflammatory phase, ED1 cells were mostly predominant, and ED2 cells increased transiently when granulation tissues were formed on PW days 3 to 7. The majority of ED1 cells expressed galectin-3. Increased number of ED1 and ED2 cells was paralleled by increased mRNA levels of TGF-β1, MCP-1 and CSF-1 on PW days 3 to 9. OX6 cells became predominant in tissue remodeling phase. These results showed that different macrophages appeared depending on progress of CWH, and that at early phases, MCP-1 and CSF-1 might recruit macrophages capable of producing galectin-3 and TGF-β1.

2.3. Cellular properties of myofibroblasts

Myofibroblasts were examined immunohistochemically with antibodies for cytoskeletons (vimentin, desmin, and α-SMA) and mesenchymal stem cell markers (Thy-1 and A3). Myofibroblasts reacting to vimentin and α-SMA began to be seen on PW day 5, then peaked on PW day 9, and gradually decreased. Desmin-reacting cells were only limited in newly-formed blood vessels. Pericytes and perifollicular dermal sheath (DS) cells in wound periphery on PW days 7 to 15 showed increased expressions for vimentin, Thy-1 and A3, although α-SMA expression was not incremented. Double immunolabeling revealed immunophenotypical similarity between myofibroblasts (vimentin+, α-SMA+, Thy-1+), pericytes (vimentin+, α-SMA+, Thy-1+, A3+), and DS cells (vimentin+, α-SMA+, Thy-1+, A3+). These findings showed that myofibroblasts might be generated from pericytes and DS cells in the lineage of mesenchymal stem cells.

Chapter 3. Immunohistochemical characterization of macrophages and myofibroblasts in rat BS

3.1. Histopathology

Scleroderma, a skin disorder characterized by persistent fibrosis, was induced in rats by daily subcutaneous injection of bleomycin (1 mg/ml) for 4 weeks (W). Samples were collected at 1 to 4 W. Dermal thickness was seen in the lower dermis at injection site, consisting of fibrosis and infiltrating macrophages. Apoptosis, demonstrable by TUNEL method, increased in hair follicles. These lesions were exacerbated with time.

3.2. Macrophages and galectin-3-expressing cells

Immunohistochemically, the numbers of macrophages reacting to ED1 and ED2 began to increase at 1 W, peaking at 2 W; the numbers gradually decreased. On the contrary, the increased number of cells reacting to OX6 was retained from 2 to 4 W. Galectin-3 expression was seen in ED1, ED2, and OX6 cells, as well as myofibroblasts. mRNA levels of

TGF-β1, MCP-1 and CSF-1 were continuously elevated. This study showed that the consistently increased numbers of macrophages, which might be induced by MCP-1 and CSF-1, could express galectin-3 and TGF-β1, thereby leading to persistent fibrosis; particularly, OX6 cells might have crucial roles in progressive fibrosis.

3.3. Immunophenotypical changes of myofibroblasts

Myofibroblasts, of which the formation was related to macrophage appearance and TGF-β1 expression, were analyzed. Myofibroblasts reacting to α-SMA, vimentin, and Thy-1 were seen in the dermis at 1 to 4 W; desmin reactivity was seen in occasional myofibroblasts. Perifollicular DS cells displayed increased reactivity for Thy-1 and vimentin, but not for α-SMA. Using double immunofluorescence labeling, both myofibroblasts and pericytes were found to express α-SMA, vimentin and Thy-1. Additionally, increased apoptosis in hair follicles was characteristically seen, being correlated with increased reactivity for vimentin and Thy-1 in perifollicular DS cells and macrophage infiltrate. Based on these findings, as mentioned in chapter 2, pericytes and DS cells might be involved in sclerodermatous fibrogenesis as possible progenitor cells of myofibroblasts.

Chapter 4.   Comparisons of properties of macrophages and myofibroblasts between CWH and BS models

In both models, ED1 and ED2 cells were predominant at early and mid stages, whereas OX6 cells appeared later. However, OX6 cells in BS were greater in number and more continuous than in CWH, suggesting that antigen-presenting cells might be important for persistent fibrosis. Galectin-3 in CWH was expressed exclusively in ED1 cells, whereas the expression in BS was seen in ED1, ED2 and OX6 cells, as well as myofibroblasts. These results suggested that activated macrophages and myofibroblasts capable of releasing galectin-3 could attribute to uninterrupted fibrosis. Interestingly, in BS, increased hair follicle apoptosis was associated with local macrophage recruitment and increased activity of myofibrobastic DS cells, of which events might lead to persistent fibrosis in this model as well.

Conclusions

1. There were differences in kinetics and distribution of macrophages with divergent immunophenotypes between epidermis, dermis, and perifollicular areas in developing rat skin.

2. CWH and BS analyses showed that macrophages exhibiting heterogeneous immunophenotypes participated in cutaneous fibrosis; ED1 and ED2 cells were predominant at early and mid stages, whereas OX6 cells appeared later.

3. Myofibroblasts in CWH and BS expressed mesenchymal markers (mainly, α-SMA, vimentin). In addition to these markers, pericytes and perifollicular DS cells reacted to stem cell markers (Thy-1 and A3), suggesting possible precursors of myofibroblasts in relation to the mesenchymal stem cell lineage.

4. There were differences in properties of macrophages and myofibroblasts between CWH and BS models; particularly, consistent increase in OX6 cells, continuous expression of galectin-3 both in macrophages and myofibroblasts followed by elevated TGF-β1, and hair follicle apoptosis accompanied by macrophage infiltrate and increased DS cell activity might have been related to persistent fibrosis in BS model.

5. Because cutaneous fibrosis models used in the present studies showed patho-morphological similarities to those in human counterpart patients, the present studies provide useful information for pathogenesis of cutaneous fibrosis, a basic pathological event, and for possible therapeutic strategies for the intractable fibrosis.

審査結果の要旨

　線維化は病理学的には組織傷害後の修復帰転であり、皮膚の創傷治癒は典型的な線維化である。しかし、慢性的な傷害が肝臓や腎臓に生じると肝硬変や萎縮腎などの難治性の線維化を招来する。線維化は、反応性マクロファージから放出される因子（特に、TGF-β1）が、コラーゲンなどの細胞外基質を産生する筋線維芽細胞を誘導することで惹起される病態である。すなわち、マクロファージと筋線維芽細胞が重要な役割を演じるが、その病理発生機序の全貌は解明されていない。

　マクロファージは、多彩な特性を有する細胞群で、浸潤マクロファージ、固着マクロファージ、抗原提示マクロファージ/樹状細胞に大別される。筋線維芽細胞は、α-平滑筋アクチン（α-SMA）を発現する拘縮細胞で、既存の線維芽細胞に由来するとされる。しかし、皮膚線維化に出現するマクロファージの多様な機能的性状、筋線維芽細胞の特性と由来については不明な点が多い。これら細胞の特性を解明し、相互の関連性を究明することは、難治性の皮膚線維化（硬化症、ケロイドなど）を治療する方策に繋がる。本研究では、異なる２つの皮膚線維化ラットモデル（パンチ創傷とブレオマイシン誘発皮膚硬化症）を確立し、線維化部位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手法を用いて解析している。得られた成績の概要は以下の通りである。

　第１章では、皮膚の発生過程（胎子、新生子、成体）における正常なマクロファージの特性を、3 種の異なるマクロファージ認識抗体を用いて免疫組織化学的に解析している。その結果、表皮においては OX6 細胞（抗原提示マクロファージ）のみが認められ、その出現は胎生期から徐々に増え始め成体で最も増加すること、真皮と毛嚢周囲では、OX6 細胞は生後から増加し始め、ED1 細胞（貪食活性マクロファージ）は胎子期からすでに著しく増加しており、ED2 細胞（炎症性因子産生マクロファージ）は新生子から徐々に増加することを見出した。さらに、真皮では ED1 細胞が、毛嚢周囲では OX6 細胞が主体となるマクロファージであることを示した。すなわち、皮膚発生過程での出現時期がマクロファージ群間で異なること、組織部位により特異的なマクロファージが出現することを明らかにした。

第 2 章では、ラットの皮膚にパンチ創傷を作成し、その後の線維化の病態（炎症、肉芽組織、修復）を経時的に解析している。その結果、ED1 細胞は炎症相で最も増加しその後減少すること、ED2 細胞は肉芽組織相で一過性に増加すること、OX6 細胞は主に修復相で出現することを示した。また、ED1 細胞が galectin-3（TGF-β1 誘導因子）を高発現すること、マクロファージ遊走関連因子（MCP-1、CSF-1）の発現が ED1 と ED2 細胞の出現と関連することを明らかにした。さらに、線維原性因子（TGF-β1）の上昇と一致して筋線維芽細胞が形成され、その筋線維芽細胞はα-SMA とビメンチンの細胞骨格マーカーに加え、組織幹細胞マーカーである Thy-1 を発現することを示した。Thy-1 発現は、新生血管周皮細胞と毛嚢周囲細胞にも認められ、これら細胞は A3 蛋白（幹細胞マーカー）も発現していた。すなわち、線維化の病態に依存し異なる特性を有するマクロファージ群が出現すること、筋線維芽細胞は血管周皮細胞や毛嚢周囲細胞に由来する可能性を示した。

　第 3 章では、ブレオマイシン誘発ラット皮膚硬化症モデルを作出し、第 2 章と同様の方法で解析している。その結果、ED1、ED2、OX6 細胞が持続的に増加すること、その出現と関連し MCP-1、CSF-1、TGF-β1 などの因子も恒常的に高発現することを明らかにした。また、進行する線維化部位にはα-SMA、ビメンチン、Thy-1 を発現する筋線維芽細胞が高頻度で出現し、血管周皮細胞とアポトーシスで萎縮した毛嚢の周囲細胞にはビメンチン、Thy-1、A3 が漸次高発現することを示した。第 2 章と同様に、進行性の線維化においても、マクロファージ群の出現と筋線維芽細胞の形成が重要であることを明らかにした。

　第 4 章では、皮膚の創傷治癒（第 2 章）と硬化症（第 3 章）モデルの線維化病変に出現するマクロファージと筋線維芽細胞の特性を詳細に解析し、比較している。その結果、硬化症では各種のマクロファージ群と誘導因子（MCP-1、CSF-1、TGF-β1）が常に高発現していること、galectin-3 の発現が、創傷治癒では ED1 細胞のみであったが、硬化症では ED1、ED2、OX6 細胞、さらに筋線維芽細胞にも発現することを見出した。また、硬化症では血管周皮細胞と毛嚢周囲細胞における筋線維芽細胞と幹細胞マーカーの発現がより増強されることも分かった。これらの違いが、皮膚硬化症における進行性の線維化に係ることを明らかにした。

　以上の研究は、皮膚線維化において、病態に依存し異なる特性を現すマクロファージ群が参画し、その出現は筋線維芽細胞の形成に係わること、さらに筋線維芽細胞の起源は組織幹細胞にあることを示唆している。線維化は、傷害に対する生体の合目的的反応であることから、本研究は、病理学的観点から生命現象を捉え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と考えられる。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。