The effects of type VI collagen on the bone formation

Summary of Doctoral Thesis

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Chapter 1: Introduction

Type VI collagen (Col VI) is a component of the extracellular matrix (ECM) in the periosteum and thought to regulate osteoblast behaviors. Several *in vitro* studies indicate that osteoblast-lineage cells required attachment to Col VI at early stages of differentiation. In addition, Col6a1-deficient mice displayed a reduction in bone mineral density and cancellous bone mass, and an aberrance of the collagen arrangement of the cortical bone. Therefore, Col VI is suggested to play an important role in normal bone formation during fetal and postnatal development. In several cell types, Col VI interacts with Neural/Glial Antigen 2 (NG2) on the cytoplasmic membrane to promote cell proliferation, spreading, and motility. However, the detailed functions of Col VI on the bone formation are still remained unclear.

The aim of this entire study is to clarify the functions of Col VI on behaviors of the osteoblast lineages and the bone formation. First of all, I propose to elucidate the spatiotemporal relationship between Col VI and osteoblast lineages expressing NG2 in the ossifying region, such as the periosteum and the groove of Ranvier (GOR) in the rat long bones during postnatal growing periods. I next investigated the effects of Col VI - NG2 interaction on the cellular behaviors of osteoblast lineages using cultured osteoblast lineages isolated from rat calvariae.

Chapter 2: Accumulation of type VI collagen in the primary osteon of the rat femur during postnatal development

In rodents, the long bone diaphysis is expanded by formation of primary osteons at the periosteal surface of the cortical bone. This ossification process is thought to be

regulated by the microenvironment in the periosteum. Col VI is a component of the ECM in the periosteum and involved in osteoblast differentiation at early stages. However, the detailed functions of Col VI and NG2 in the ossification process in the periosteum are still under investigation. In this chapter, to clarify the spatiotemporal relationship between Col VI-NG2 interaction and formation of the primary osteon, I examined the distribution of Col VI and osteoblast lineages expressing NG2 in the periosteum of rat femoral diaphysis during postnatal growing periods by immunohistochemistry. Primary osteons enclosing the osteonal cavity were clearly identified in the cortical bone from 2 weeks of age. The size of the osteonal cavities decreased from the outer to the inner region of the cortical bone. In addition, the osteonal cavities of newly formed primary osteons at the outermost region started to decrease in size after rats reached the age of 4 weeks. Immunohistochemistry revealed concentrated localization of Col VI in the ECM in the osteonal cavity, but not in the osteogenic layer of the periosteum. Col VI-immunoreactive areas were reduced and disappeared as the osteonal cavities became smaller from the outer to the inner region. In the osteonal cavities of the outer cortical regions, Runt-related transcription factor 2 (RUNX2)-immunoreactive spindle-shaped cells and mature osteoblasts were detected in Col VI-immunoreactive areas. The numbers of RUNX2-immunoreactive cells were significantly higher in the osteonal cavities than in the osteogenic layers of the periosteum from 2 to 4 weeks. Most of these RUNX2-immunoreactive cells showed NG2-immunoreactivity. Furthermore, PCNA-immunoreactivity was detected in the RUNX2-immunoreactive spindle cells in the osteonal cavities. These results indicate that differentiation and proliferation of the osteoblast lineage occur in the Col

VI-immunoreactive area. Thus, Col VI may provide a characteristic microenvironment for regulation of the osteoblast lineage behavior in the osteonal cavity of the primary osteon. Interaction of Col VI and NG2 may be involved in the structural organization of the primary osteon by regulating osteoblast lineages.

Chapter 3: Distribution of type VI collagen in the Groove of Ranvier during rat postnatal development

The Groove of Ranvier (GOR) is thought to be the ossification area situated around the growth plate cartilage. The inner layer of the GOR consists of mature osteoblasts on the surface of the bone bark, thin trabecular bone of the epiphyseal tip of the cortical bone, around the epiphyseal growth plate. In addition, the middle layer containing undifferentiated mesenchymal cells provides mesenchymal stem cell niche. These lines of evidence indicate that early-stage osteoblast lineages differentiate to mature osteoblasts in the GOR, which form new bone tissues at the epiphyseal region of the cortical bone, resulting in longitudinal growth of the cortical bone. In this chapter, to clarify the spatiotemporal association of Col VI with osteoblast differentiation in the GOR, I examined the distribution of Col VI and osteoblast lineages expressing NG2 in the rat tibia proximal end during postnatal growing periods by immunohistochemistry. Col VI-immunoreactivity was detected in the upper middle layer, but not in the inner and lower middle layer. RUNX2 + / Osterix (OSX) – osteoblast lineages were detected in Col VI-immunopositive areas. However, RUNX2 + / OSX + mature osteoblasts were only found in the Col VI-immunonegative area. Most of the RUNX2 + cells showed NG2-immunoreactivity. These findings indicate that Col VI provided a characteristic

microenvironment for differentiation of the osteoblast lineages prior to terminal differentiation in the GOR. Col VI may regulate the differentiation by interaction with NG2 expressed on the osteoblast lineages.

Chapter 4: The effects of type VI collagen on the osteoblastic behavior

The results of Chapter 2 and 3 raised the possibility that Col VI interaction regulates proliferation and differentiation of the osteoblast lineages prior to terminal maturation to the mature osteoblast producing bone matrix. In this chapter, to address this hypothesis, I investigated effects of Col VI on the behaviors of osteoblast lineages using osteoblasts isolated from rat calvariae cultured on Col VI-coated dish. The proliferation of the osteoblasts was significantly decreased on Col VI-coated dish compared to cells on non-coated dish. In the migration assay, Col VI enhanced haptotaxis and motility of the osteoblasts. In the examinations of expression of differentiation markers by quantitative real time RT-PCR, OSX mRNA was decreased in the osteoblasts on Col VI-coated dish at 10 and 15 days after differentiation induction (Day 10 and 15), whereas RUNX2 mRNA expression was not affected during the entire culture period. Expressions of osteocyte markers, such as Dentin matrix protein 1, Sclerostin, and Receptor activator of nuclear factor kappa-B ligand, were significantly decreased in the osteoblasts on Col VI at Day 15. As for bone matrix production, Osteocalcin mRNA expression and mineralization were significantly inhibited in the osteoblast on Col VI at Day 10 and 15. However, Osteopontin (OPN) mRNA was significantly increased in the osteoblast on Col VI at Day 5 and 10. In the differentiation process of osteoblast lineages, OSX promotes differentiation of the osteoblast lineage at the later phases, and OPN is a

negative-regulator of osteoblast proliferation, differentiation, and mineralization. Thus, the results of this study indicate that Col VI suppresses differentiation of the osteoblast lineage and mineralized bone matrix production especially at later phases via inhibition of OSX expression and increase of OPN expression.

Chapter 5: Interactions between Notch1 and DLL1 in the rat femur primary osteon during postnatal development

Notch signaling is one of major negative regulators in osteoblast differentiation and bone formation by inhibition of RUNX2 transcriptional activity. The results of Chapter 4 demonstrated inhibition of differentiation of the osteoblast lineage by Col VI, indicating that Notch signaling is involved in the inhibition pathway induced by Col VI. However, the detailed functions of Notch signaling in the Col VI-associated regulation of the osteoblast lineage are still under investigation. Furthermore, it is also unclear whether Notch signaling regulates the primary osteon formation. In this chapter, to clarify the spatiotemporal relationship between Notch signaling and formation of the primary osteon, I examined the distribution of osteoblast lineages expressing Notch1, activated Notch1 (NICD) and Delta-like ligand 1 (DLL1) in the periosteum of rat femoral diaphysis during postnatal growing periods by immunohistochemistry. I also examined the expressions of Notch1 and DLL1 mRNA in osteoblasts on Col VI-coated dish to determine whether Col VI regulates Notch signaling in the osteoblast lineage using primary culture of osteoblasts. Immunohistochemistries of the primary osteon revealed that Notch1, NICD, and DLL1 were restricted to the RUNX2-positive osteoblast lineages in the osteonal cavity. Thus, Notch signaling may associates with

formation of the primary osteon via down-regulation of the osteoblast differentiation. In the cultured osteoblast lineages on Col VI, Notch1 and DLL1 mRNAs are significantly increased at Day 5 compared with control culture. These findings indicate that Col VI stimulates Notch1 and DLL1 expression in the immature osteoblast, causing the inhibition of osteoblast differentiation.

In conclusion, this study revealed that Col VI provide characteristic microenvironment for the ossification of the cortical bone in the primary osteon and the GOR. Immature osteoblast lineages were detected in the Col VI positive area, while mature osteoblasts in the Col VI negative area, indicating that Col VI regulates differentiation of the osteoblast lineages prior to terminal differentiation. These osteoblast lineages express NG2, indicating that the interaction between NG2 and Col VI may regulate the differentiation of osteoblast lineages. Additionally, *in vitro* study indicates that Col VI inhibits osteoblast maturation/differentiation and bone matrix production via inhibition of OSX expression, and increase of OPN expression and Notch signaling. These findings indicate that Col VI inhibits osteoblast differentiation, leading to regulation of cortical bone formation in the primary osteon and the GOR during postnatal growing periods.