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Form 3

Abstract of Thesis

Name (LI AONAN)	
Title	Role of heparan sulfate in vasculogenesis of dental pulp stem cells （歯髄幹細胞の脈管形成におけるヘパラン硫酸の役割）

[Objective]

Dental pulp stem cells (DPSCs) can differentiate into vascular endothelial cells and display sprouting ability. During this process, cell responses to the extracellular microenvironment and cell-extracellular matrix interactions are critical in determining stem cell fate. Heparan sulfate (HS) glycosaminoglycan is known as a widely distributed constituent of extracellular matrix in all mammal tissues. HS could enhance the binding affinity between growth factors and related receptors, subsequently modulating a variety of biological activities. However, the regulatory function of HS in the vasculogenesis of mesenchymal stem cells remains unclear. In this study, it was hypothesized that HS highly orchestrates endothelial differentiation of DPSCs and their subsequent formation of nascent blood vessels. The purpose of this study was to investigate the role of HS in endothelial differentiation and vasculogenesis of DPSCs.

[Materials and Methods]

EXPERIMENT 1. Vasculogenic behaviors of HS antagonist-treated DPSCs

DPSCs were cultured in DMEM containing 20% FBS and 1% penicillin/streptomycin, which was designated as growth medium (GM). To induce endothelial differentiation, DPSCs were cultured in endothelial differentiation medium (EM) consisting of endothelial cell growth medium (EGM2-MV) supplemented with 50 ng/mL $rhVEGF_{165}$. An HS antagonist, surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide), was used to inhibit the production of HS in DPSCs. DPSCs were cultured with GM or EM in the absence or presence of 1, 5, or 10 µM surfen. After 7 days, cell proliferation was examined by WST-8 assay. In addition, the expressions of pro-angiogenic markers, *VEGFA* and *CXCL1*, and stemness marker *Nanog* in DPSCs under surfen treatment were evaluated by real-time PCR.

To visualize the sprouting ability and cell viability of DPSCs under the inhibition of HS production, cells were seeded onto the Matrigel-precoated plate and maintained in EM with or without surfen. After incubation for up to 14 days, the reticular-like structure formed by DPSCs was observed by light microscope, and quantification analysis was conducted by ImageJ. In addition, DPSC viability in 3D culture was assessed by live/dead staining.

EXPERIMENT 2. Vasculogenic behaviors of *EXT1*-silenced DPSCs

Establishment of EXT1-silenced DPSCs: Exostosin1 (EXT1), a crucial glycosyltransferase for HS biosynthesis, was silenced by transfecting with short hairpin RNA specific for *EXT1*. DPSCs transduced with scrambled sequences were used as control. These cells were cultured with EM for up to 14 days, and gene silencing efficiency and HS production were evaluated.

Gene expressions: *EXT1*-silenced DPSCs and control DPSCs were cultured with EM for 14 days. Total RNA was extracted for RNA sequencing. The expressions of *VEGFA*, *CXCL1*, and *Nanog* in both cells were further evaluated by real-time PCR.

Vasculogenic behaviors: Cells were seeded on the Matrigel-precoated plate and divided into 3 groups. Group 1: control DPSCs, group 2: *EXT1*-silenced cells, and group 3: *EXT1*-silenced cells supplemented with 100 µg/mL HS. After 7 and 14 days of culture with EM, the sprouting ability of DPSCs was evaluated using microscopic images.

EXPERIMENT 3. Impact of HS on vascular formation *in vivo*

The biodegradable poly-L-lactic acid (PLLA) scaffolds seeded with *EXT1*-silenced or control DPSCs were implanted into the subcutaneous space of six-week-old male severe combined immunodeficiency (SCID) mice. After 5 weeks, PLLA scaffolds were retrieved and prepared for histological evaluation.

[Results and Discussion]

EXPERIMENT 1.

Cell proliferative capacity under endothelial differentiation was suppressed by increasing concentrations of surfen; however, no significant difference was detected for cells cultured in GM. Expression of pro-angiogenic markers *VEGFA* and *CXCL1* significantly declined with increasing dosages of surfen; in contrast, expression of stemness marker *Nanog* increased at day 7 ($p < 0.05$).

The sprouting property of DPSCs was suppressed by the addition of surfen. Image analysis revealed that numbers and total length of sprouting branches were significantly decreased in DPSCs cultured with increasing dosages of surfen ($p < 0.05$). Live/dead staining showed that dead cells increased by the surfen stimulation, however, there were less dead DPSCs compared with the areas occupied by living cells. These results demonstrated that HS antagonist hampered sprouting capacity of DPSCs without affecting cell viability.

EXPERIMENT 2.

Establishment of EXT1-silenced DPSCs: The mRNA and protein levels of EXT1 expression were significantly reduced in *EXT1*-silenced DPSCs after 7 and 14 days of induction, suggesting that EXT1 was successfully downregulated ($p < 0.05$). Additionally, *EXT1* silencing led to lower production of HS compared with control DPSCs ($p < 0.05$).

Gene expressions: EXT1 deficiency significantly altered the gene expression profile in DPSCs. Expression of *VEGFA* and *CXCL1* was downregulated in *EXT1*-silenced cells, while *Nanog* expression increased ($p < 0.05$). These results indicated that a lack of HS inhibited endothelial differentiation potential and maintained the stemness property of DPSCs.

Vasculogenic behaviors: After 7 days of endothelial induction, *EXT1*-silenced DPSCs exhibited less sprouting and formed fewer reticular-like structures compared with control DPSCs. Notably, the addition of exogenous HS ameliorated the sprouting capacity of *EXT1*-silenced DPSCs ($p < 0.05$).

EXPERIMENT 3.

Immunofluorescence staining showed that control DPSCs formed blood-containing vessels positive for human-specific CD31 and von Willebrand factor, indicating that vascular endothelial cells originating from DPSCs formed nascent blood vessels and anastomosed with the host vasculature. In addition, numbers of blood-containing vessels were significantly smaller in the scaffolds seeded with *EXT1*-silenced DPSCs compared with specimens loaded with control DPSCs ($p < 0.05$). These results demonstrated that EXT1 plays an important role in vascular formation originating from DPSCs, and HS is essential for modulating vasculogenic processes of DPSCs.

[Conclusion]

This study demonstrates that HS is required for the regulation of endothelial differentiation and vasculogenesis of DPSCs, suggesting the possible usefulness of application of HS glycosaminoglycan to dental pulp regeneration and tissue engineering.

様式７

論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

本研究は、グリコサミノグリカンの一つであるヘパラン硫酸が歯髄幹細胞の血管内皮細胞分化および脈管形成に及ぼす影響を評価したものである。

その結果、*in vitro* の実験系において、ヘパラン硫酸の拮抗薬の添加およびヘパラン硫酸の糖転移酵素（*EXT1*）のノックダウンにより歯髄幹細胞の血管内皮細胞への分化が抑制され、*EXT1* をノックダウンした歯髄幹細胞培養系にヘパラン硫酸を添加すると、血管内皮細胞への分化能が回復することが明らかとなった。さらに、*in vivo* 実験により、*EXT1* をノックダウンすると歯髄幹細胞の脈管形成能が低下することを確認した。

以上の研究成果は、ヘパラン硫酸が歯髄幹細胞の脈管形成能の発現に重要な役割を果たしていることを示すものであり、本研究は博士（歯学）の学位授与に値するものと認める。