主　論　文

Exogenous DKK-3/REIC inhibits Wnt/β-catenin signaling and cell proliferation in human kidney cancer KPK1

（腎がん細胞における外因性タンパクDKK-3/REICはwnt/β-catenin経路と細胞増殖を抑制する）

[緒言] Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults, accounting for about 3% of all adult malignancies and it's the seventh-most common cancer in males, the nint-most common cancer in females, and represents approximately 90% of all kidney malignancies.

Wnt signaling plays an important role in development of diseases in adults. Wnt triggers at least two, likely three, pathways: the first is the canonical Wnt/β-catenin pathway, in which Wnt3a, a member of the Wnt ligands, binds to LRP6. This results in the stabilization of cytosolic β-catenin and facilitates its translocation into the nucleus to activate downstream transcription factors. The second pathway involved is the planar cell polarity (PCP) pathway, which does not involve β-catenin.. The final possible pathway, the Wnt/Ca2+ cascade, is still largely controversial and may overlap in part with the PCP pathway.

Dickkopf (DKK) genes comprise an evolutionary conserved gene family of four members (DKK 1-4). Exogenous DKK 1, 2 and 4 are reported to possess similar functions in suppressing the activity of Wnt/β-catenin (canonical) pathway by binding to the co-receptor LRP5/6 with high affinity. However, DKK-3, alternatively known as reduced expression in immortalized cells (REIC), does not seem to bind to LRP5/6 or inhibit the Wnt/β-catenin signaling, as has been declared in many research papers.

We prepared exogenous DKK-3 protein and investigated the effects on KPK1 human renal carcinoma cells and tested the anti-proliferative effects. To determine whether or not exogenous DKK-3 inhibits Wnt/β-catenin signaling, we investigated DKK-3 protein on the levels of phosphorylated and non-phosphorylated β-catenin in KPK1 cells. We also examined the involvement of LRP6 transmembrane receptor in the extracellular actions of DKK-3 protein and in Wnt/β-catenin signaling.

[材料と方法] Materials and methods

Cell culture

The human renal cell KPK1 was provided by Professor S. Naito. Human prostate cancer cell PC3 and human malignant mesothelioma cell 211H were obtained from the American Type Culture Collection. The cells were grown in DMEM medium supplemented with 10% (v/v) FBS, penicillin (100 IU/ml) and

streptomycin (100 μg/ml). The cells were cultured at 37 °C in the atmosphere of 5% CO_2 and air and split at a 1:5 ratio every 3 days.

Preparation of the recombinant DKK-3 protein

The recombinant protein of human full length DKK-3 was transiently expressed in FreeStyle™ 293-F cells using Freestyle 293 Expression Medium and the 293Fectin transfection reagent under the manufacturer's instructions. Cells (1×10^6 cells/ml) with 180 ml medium were prepared in a 500-ml flask. After transfection with 180 μg each of the expression plasmid DNA and 293Fectin complex, the cells were cultivated using an orbital shaker (125 rpm) at 37 °C in the presence of 8% CO_2 for 4 days. The secreted proteins in the culture medium were concentrated by Amicon Ultra centrifugal filter units and purified and stored as previously described.

Western blot analysis

The cells were treated with the DKK-3 protein at a final concentration of 10 μg/mL in the culture medium, and the cell lysate was obtained before treatment and at 1, 6, 24, 48 and 72 h after treatment. The cells were collected in ice-cold lysis buffer for 15 min on ice. The cell fragments were removed by centrifugation, and the supernatant lysate was immediately stored at -80 °C. The protein concentrations were determined using a protein assay kit. After then adding 5x loading buffer, the extracted cell protein (10 μg) was separated using a Mini-Protean TGX gels kit. The proteins on the gel were transferred onto a PVDF membrane using a Trans-Blot Turbo Transfer System. After the transfer, the membrane was blocked in 5% non-fat milk with TBS-T buffer and then incubated with primary antibodies overnight at 4 °C. After washing the membrane with TBS-T 3 times, the membrane was incubated with secondary antibodies with 5% BSA in TBS-T buffer for 1 h. The protein-antibody complexes were visualized by the enhanced chemiluminescence detection method using medical X-ray film. All antibodies were purchased from Cell Signaling Technology, Inc.

Cell proliferation assay

KPK1 cells were plated in 96-well plates at a final volume of 100 μL culture medium. After 12-h incubation, the cells were treated with the DKK-3 protein at a final concentrations of 10 or 50 μg/mL in culture medium. PBS was added at the same volume as control. The cell viability was assayed at the indicated days after treatment using a Cell Proliferation Kit XTT II at an absorbance of 492-690 nm.

siRNA transfection for LRP6 knockdown

KPK1 cells (2×10^5 cells per well in 6-well plates) were incubated with 2 ml antibiotic-free culture medium supplemented with FBS for 18-24 h until the cells were 60%-80% confluent. The LRP6 siRNA and control reagent were transfected into KPK1 cells for 6 h at 37 °C in a CO_2 incubator. After transfection, the medium was changed to the complete medium and further incubated for 24 h. The cells

were then treated with the recombinant DKK-3 protein at a final concentrations of 10 μg/mL in culture medium, and the cell lysate was obtained for a Western blot analysis at the indicated time points.

Statistical analyses

The data are shown as the means ± standard error. Unpaired Student's *t*-tests were performed for the statistical analyses. Differences were considered to be statistically significant at $p < 0.05$.

［結果］ **Results**

Exogenous DKK-3 inhibits Wnt/β-catenin signaling in a time-dependent manner in KPK1 cells.

The phosphorylated β-catenin (inactive form) was gradually increased in KPK1 cells in a time-dependent manner. The level of non-phosphorylated β-catenin (activated form) was gradually reduced, indicating that exogenous DKK-3 protein inhibits Wnt/β-catenin signaling in a time-dependent manner. We further elucidate the inhibitory mechanism, the level of phosphorylated GSK-3β was gradually increased as the phosphorylated β-catenin was increased. As for the PC3 and 211H cells, the level of phosphorylated β-catenin was extremely weak and not increased in the time course treated with DKK-3 protein. In these cells, on the contrary to KPK1 cells, the level of phosphorylated GSK-3β was gradually decreased.

Exogenous DKK-3 inhibits the downstream targets of Wnt/β-catenin signaling.

The downstream key effectors of Wnt/β-catenin signaling, the level of two key targets, known as tumor progression factors, was significantly suppressed after treatment. The TCF1 expression was markedly reduced at an early phase after DKK-3 treatment and subsequently restored. the c-Myc was gradually reduced after treatment. The expression of Wnt3a was also decreased in a time-dependent manner. In contrast, no significant changes in the expression of Met. These findings indicate that exogenous DKK-3 protein down-regulates the Wnt/β-catenin-targeted molecules of TCF1 and c-Myc.

Exogenous DKK-3 exhibits anti-proliferative effects in KPK1 cells.

The viability of cells treated with the final concentrations of 10 or 50 μg/mL of DKK-3 protein was measured, and significant inhibition of cell proliferation was observed in both cases at day 4. Dose dependency was observed in the inhibitory effects.

LRP6 knockdown elevated the base level of the Wnt/β-catenin signaling but did not influence the up-regulation course or pattern.

We silenced the LRP6 gene with siRNA and confirmed the expressional depletion by a Western blot analysis. Under a lack of LRP6 expression, we analyzed the effects of DKK-3 on the time course of the

phosphorylated β-catenin and c-Myc expression. However, LRP6 depletion up-regulated the base level of the phosphorylated β-catenin expression, maintaining its up-regulation course and pattern of control. However the c-Myc base level was attenuated, maintaining its down-regulation course and pattern. The depletion of the Wnt pathway receptor LRP6 exerted no significant effects on the extracellular DKK-3-induced course of Wnt/β-catenin signaling, indicating that DKK-3 blocks the signaling in a LRP6-independent manner in KPK1 cells.

[考察] Discussion

DKK-3 is a tumor suppressor in a variety of cancer cells; however, the role and mechanism of exogenous DKK-3 protein in the Wnt/β-catenin signaling pathway has remained unclear. In addition, whether or not exogenous DKK-3 inhibits Wnt signaling, as well as the exogenous DKK-1 and DKK-2 of inhibitors of the Wnt signaling is also unclear (13). In the present study, we demonstrated that the level of phosphorylated β-catenin was increased in KPK1 cells treated with exogenous DKK-3 protein. The non-phosphorylated β-catenin was consistently decreased, clearly indicating that the DKK-3 protein works as an inhibitor of Wnt/β-catenin on the cell surface.

Extremely few studies have described the involvement of extracellular DKK-3 protein in Wnt/β-catenin signaling. In DKK-3-transfected human glioma cells, a decrease in the non-phosphorylated β-catenin (activated form of β-catenin) was observed in parallel with the increase in the intra- and extracellular DKK-3 protein. The authors suggested that the extracellular DKK-3 inhibited the Wnt/β-catenin signaling pathway via binding to the transmembrane receptors. The findings of previous studies showing a lack of modulation by extracellular DKK-3 protein on the Wnt/β-catenin pathway as well as the results of the current study suggest that the role of DKK-3 may be based on the cell type and characteristics.

We revealed that expression of TCF1 and c-Myc, the downstream transcription factors of Wnt/β-catenin signaling in cancer transcription cascades, are down-regulated by treatment with the DKK-3 protein. These results not only support the suppressive effects of exogenous DKK-3 protein on Wnt/β-catenin signaling but also suggest the inhibitory role of DKK-3 in the cell proliferation promoted by TCF1 and c-Myc. Indeed, a cancer cell viability test confirmed the anti-proliferative effects of exogenous DKK-3 protein in the KPK1 cells, probably due to the suppressed levels of TCF1 and c-Myc in Wnt/β-catenin signaling. Interestingly, the significant down-regulation of TCF1 was observed early (6 h) after treatment, and the expression of c-Myc gradually decreased, as shown Figure 2. Since TCF1 is a transcription factor for c-Myc expression, the down-regulation of TCF1 likely induced the gradual decrease in the c-Myc level. We also examined the levels of Met protein, which is regulated by transcription factors other than TCF1. There were no significant changes in the Met expression after treatment with recombinant DKK-3 protein, suggesting that its regulation is independent of the exogenous DKK-3 related modification of Wnt/β-catenin signaling. As for the reduced expression of Wnt3a after DKK-3 treatment, modifications of the activities of the β-catenin/TCF1 target genes may have transcriptionally suppressed its transcription. The down-regulation of these molecules may be due to transcriptional suppression through the Wnt/β-catenin signaling pathway affected by exogenous DKK-3 protein.

Because LRP6 is a cell-surface receptor of extracellular DKK-1 and DKK-2 proteins and such interaction inhibits Wnt/β-catenin signaling, we examined whether or not exogenous DKK-3 protein

affects the LRP6-mediated Wnt/β-catenin signaling in KPK1 cells. We silenced the LRP6 gene and subsequently analyzed the effects of DKK-3 on the time course of the up-regulation of phosphorylated β-catenin expression. Interestingly, LRP6 depletion elevated the base level of the phosphorylated β-catenin expression; however, there was no effect on its up-regulation course or pattern. With regard to the downstream c-Myc, the base level declined but maintained its down-regulation course and pattern. The involvement of extracellular DKK-3 in the Wnt/β-catenin signaling pathway may function as an alternative mechanism rather than as an antagonist blocking LRP6 in KPK1 cells. Given a previous report that DKK-3 interacts with the Wnt pathway receptors Kremen1 (Krm1) and Kremen2 (Krm2) and not with LRP6 in biochemical assays, it is worth further exploring the modification of Wnt/β-catenin signaling in terms of the interaction of DKK-3 with the Krm receptors on cell surface.

In order to analyze in the other cell types than KPK1 cells, we examined the exogenous DKK-3 related modification of Wnt/β-catenin signaling in human prostate cancer PC3 cells and human malignant mesothelioma 211H cells. The treatment with recombinant DKK-3 protein did not lead to elevation of phosphorylated β-catenin in the PC3 cells and 211H cells. The results indicate that the inhibitory effect of DKK-3 on Wnt/β-catenin signaling could be absent or masked. We also investigated the effect of exogenous DKK-3 protein on the level of phosphorylated GSK-3β, the possible upstream regulator of phosphorylated β-catenin in Wnt/β-catenin signaling. Unexpectedly, the effect on the level of phosphorylated GSK-3β was opposite between the human kidney cancer KPK1 cells and the other two cancer cells. Further study could elucidate the molecular mechanisms that exogenous DKK-3 protein modifies the level of phosphorylated β-catenin in the GSK-3β/β-catenin axis.

[結論] Conclusion

In summary, we demonstrated for the first time that exogenous treatment with DKK-3 protein exerts anti-proliferative effects in KPK1 cells and inhibits the Wnt/β-catenin signaling in a LRP6-independent manner. Exogenous DKK-3 protein seems to inhibit the proliferation of KPK1 cells by inactivating Wnt/β-catenin signaling with up-regulation of phosphorylated β-catenin. Although the binding partner of DKK-3 on the cell surface remains to be elucidated, these findings help clarify the anti-cancer mechanisms of extracellular DKK-3. The Wnt signaling pathway plays an important role both in the carcinogenesis and progression of renal cell carcinoma (23), and we previously demonstrated the *in vivo* tumor-suppressive effects of the recombinant full length DKK-3 protein in a tumor model of murine renal carcinoma (RENCa) (24). Recombinant DKK-3 protein may be a promising agent for treating renal cell carcinoma.