Promotion of the Self-renewal Capacity of Human Acute Leukemia Cells by Wnt3A

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Abstract. *Background: Wnt/β-catenin signaling is involved in the growth of various types of cancer cells. Wnt3A has been reported to promote the self-renewal of hematopoietic stem cells. Materials and Methods: The effects of recombinant Wnt3A protein on the in vitro growth of four acute myeloid leukemia (AML) and four acute T-lymphoblastic leukemia (T-ALL) cell lines was examined. Results: Wnt3A stimulation either had no effect on, or slightly suppressed, the short-term growth of these cell lines. In three cell lines, Wnt3A promoted clonogenic cell recovery after suspension culture, suggesting the promotion of the self-renewal capacity of leukemic stem or progenitor cells. Immunoblot analysis showed that Wnt3A stimulation reduced phosphorylated β-catenin and increased β-catenin in these cells, indicating that Wnt3A stimulation activated Wnt/β-catenin signaling. Conclusion: Wnt3A stimulation did not promote the growth of whole cell populations, but did promote the self-renewal of leukemic stem/progenitor cells in some AML and T-ALL cell lines.*

The Wnt/β-catenin signaling pathway plays a role in regulating the self-renewal of hematopoietic stem cells (1, 2). This signaling has also been implicated in the progression of various types of cancer, such as colon cancer and leukemia (3). In the latter case, its involvement has been reported in acute myeloid leukemia (AML) (4-6), acute lymphoblastic leukemia (ALL) (7), chronic myelogenous leukemia (8) and chronic lymphocytic leukemia (9).

The signaling is activated by binding of Wnt proteins to receptors. In the absence of a signal, a complex of proteins containing glycogen synthase kinase 3β (GSK3β) phosphorylates β-catenin. The phosphorylated β-catenin is degraded by proteosomes. When the Wnt signal is activated, the activity of GSK3β is inhibited, resulting in the accumulation of β-catenin. The accumulated β-catenin translocates to the nucleus and induces the expression of growth-related genes (3). The human Wnt family consists of 19 members. Among them, Wnt3A is reported to be important for hematopoiesis (2).

Recently, the effects of Wnt3A protein on the *in vitro* growth of B-ALL cells has been reported by two groups (10, 11). One group showed that Wnt3A inhibited the proliferation of B-ALL cells, whilst the other showed that it promoted their growth. The reason for this discrepancy is not clear.

In this paper, we examined the effects of recombinant Wnt3A protein on the growth of AML and T-ALL cells in culture. We focused particularly on its effects on the self-renewal capacity of the cells because Wnt signaling is thought to be important in leukemic stem cells. To our knowledge, this is the first report to show the effects of Wnt3A on the self-renewal of AML and T-ALL cells.

Materials and Methods

Cells and reagents. Four human AML cell lines, OCI/AML3 (established at Ontario Cancer Institute, Canada), TMD7 (12), HEL and THP-1 (supplied by the Japanese Cancer Research Resources Bank) and four T-ALL cell lines, Jurkat, KOPT-K1, DND-41 and ALL-SIL (gift from Drs. Harashima and Orita, Fujisaki Cell Center, Japan) were used. Recombinant mouse Wnt3A was purchased from R & D Systems (Minneapolis, USA). A GSK3 inhibitor, SB216763, which mimics the activation of Wnt/β-catenin signaling, was purchased from Sigma (St. Louis, USA).

Short-term growth assay. The effects of Wnt3A on short-term growth was examined using a colorimetric assay (WST-1 assay). Cells (0.2-1×10^4 cells/well) were cultured in 0.1 ml of 2% fetal calf serum-supplemented RPMI-1640 medium (Gibco BRL, USA) with increasing concentrations of Wnt3A (0-100 ng/ml). After 3 days, a solution containing WST-1 and 1-methoxy PMS (Dojindo Laboratories, Japan) was added. After incubation, the optical density, which provided an indication of cell number, was measured using an ELISA reader.

Colony assay. The effects of Wnt3A on colony formation by leukemia cells were examined using methylcellulose culture. In 96-well culture plates, 0.2-1×10^4 cells/well were plated in 0.1 ml of

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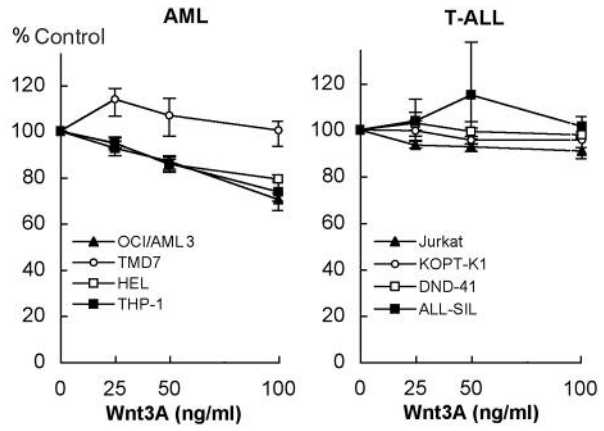

Figure 1. *Dose-response curves showing the effects of Wnt3A on the growth of AML and T-ALL cell lines by WST-1 assay. Cells were cultured with increasing concentrations of Wnt3A. After 3 days, the optical density (OD) was measured. Growth is shown as a percentage of the mean OD value of control cells cultured without Wnt3A.*

Table I. *Effect of Wnt3A on colony formation and clonogenic cells recovered (CCR) after suspension culture.*

Cell	Colony assay		Suspension culture followed by colony assay					
	Mean colony no.		Cell no. ($\times10^5$)		PEs ($\times10^{-2}$)		CCR ($\times10^4$)	
	Cont	Wnt	Cont	Wnt	Cont	Wnt	Cont	Wnt
OCI/AML3	202	180	5.5	4.5	3.1	6.1	1.7	2.8*
TMD7	40	43	1.9	1.9	2.0	1.9	0.38	0.36
HEL	255	302*	2.3	2.3	4.9	4.3	1.1	0.99
DND-41	131	120	3.0	3.0	2.9	4.0	0.87	1.2*
Jurkat	94	100	1.3	1.4	2.1	2.8	0.27	0.39*

PEs is the plating efficiency after suspension culture. CCR value is the product of the cell number and PEs. Asterisks represent statistically significant differences between the control cells and Wnt3A-stimulated cells ($p<0.05$).

RPMI-1640 medium containing 0.8% methylcellulose, with or without Wnt3A (50 ng/ml). After 7 days' culture, colonies containing more than 20 cells were counted under an inverted microscope. Student's *t*-test was used to determine statistical significance of differences between treatments.

Self-renewal capacity. The self-renewal capacity of leukemia cells was evaluated by the recovery of clonogenic cells after suspension culture as described elsewhere (13). In 24-well culture plates, 1×10^5 cells were cultured in 1 ml of RPMI-1640 medium with or without Wnt3A (50 ng/ml). After 5 days, the cells were harvested and the cell numbers were counted. The harvested cells were then plated on 0.1 ml of methylcellulose-containing RPMI-1640 medium without Wnt3A. After 7 days' culture, colony numbers were counted. The plating efficiency following suspension culture (PE_s) was evaluated as the mean colony number/10^4 cells. The clonogenic cells recovered (CCR) were calculated by multiplying the number of harvested cells by the PE_s.

Cell morphology. To examine the effects of Wnt3A on differentiation, cells cultured with Wnt3A were observed under an inverted microscope. Cytospin preparations were then created from harvested cells and stained with Wright-Giemsa.

Flow cytometric analysis. It has been reported that CD34-positive and CD38-negative fractions contain leukemic stem cells (14). We therefore examined these fractions in cells stimulated with Wnt3A for 24 h, using anti-CD34, -CD38 and control antibodies (BD Biosciences, USA).

Immunoblotting. To examine whether or not Wnt3A stimulation activated the Wnt/β-catenin pathway, immunoblot analysis was performed. Cells were harvested and lysed before and after culture with Wnt3A (50 ng/ml) or SB216763 (5 μM) for 3 h. SB216763 was used as a positive control to inhibit phosphorylation of β-catenin. The lysates from 1×10^6 cells/lane were subjected to SDS-PAGE and immunoblotted with anti-phosphorylated β-catenin, anti-β-catenin (Santa Cruz Biotechnology, USA), anti-cleaved Notch1 (Cell Signaling Technology, USA) (to selectively detect the active form of Notch1 protein), and anti-β-actin (Abcam, UK) antibodies. Immunoreactive bands were detected with an ECL kit (GE Healthcare, UK). The experiments were repeated at least twice to verify their reproducibility.

Quantitative RT-PCR. Wnt activation has been reported to induce expression of *NOTCH1* (1) and *JAGGED1* mRNA (15). We therefore examined the effects of Wnt3A stimulation on the expression of these genes. Cells were cultured with or without 50 ng/ml Wnt3A for 24 h. Total RNA was extracted and first-strand cDNA was synthesized. Quantitative PCR was performed using a FastStart DNA Master SYBR Green I kit and LightCycler primer sets for *NOTCH1*, *JAGGED1* and *β-ACTIN* (Roche Diagnostics, Germany), as reported previously (16).

Results

Effect of Wnt3A on short-term cell growth. The dose response curves showing the effects of Wnt3A on short-term growth of AML and T-ALL cells are shown in Figure 1. In three AML cell lines, OCI/AML3, HEL and THP-1, Wnt3A stimulation slightly suppressed cell growth. In T-ALL cells, the growth was not significantly affected by Wnt3A. Each assay was repeated three times independently to verify its reproducibility.

Effect of Wnt3A on colony formation and self-renewal capacity. The effects of Wnt3A on colony formation are shown in the left-hand column in Table I. Wnt3A stimulation slightly increased the number of colonies of HEL. Colony formation was not significantly affected in the remaining four cell lines shown in the Table. The other three cells lines (not shown in Table I) proliferated diffusely in methylcellulose, therefore we could not count the colony numbers.

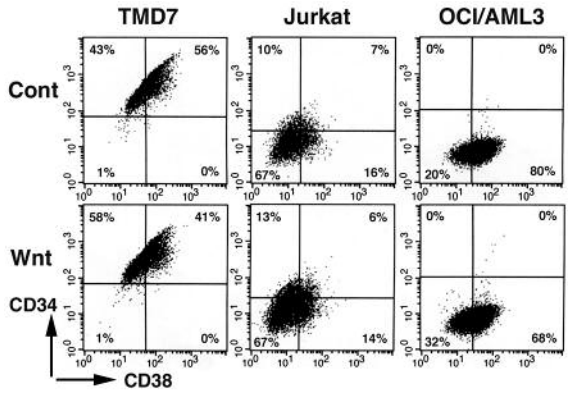

Figure 2. *Flow cytometric analysis of CD34 and CD38 expression in cells cultured with or without Wnt3A for 1 day.*

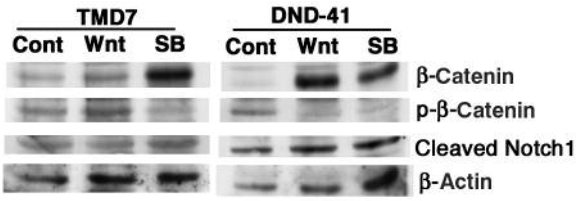

Figure 3. *Effects of Wnt3A stimulation on β-catenin and Notch1 proteins. Cells were harvested and lysed before (Cont) and after culture with Wnt3A (Wnt) or SB216763 (SB) for 3 h. The lysates from 1×10^6 cells/lane were subjected to SDS-PAGE and immunoblotted with anti-β-catenin, anti-phosphorylated β-catenin, anti-cleaved Notch1 and anti-β-actin antibodies.*

The right-hand column in Table I shows the CCR as an indicator of self-renewal capacity. In OCI/AML3, DND-41 and Jurkat cells, the CCR was promoted by Wnt3A stimulation. In TMD7 and HEL cells, the CCR was not significantly affected by Wnt3A.

Effect of Wnt3A on morphology and surface antigens. We examined the cytospin preparation stained with Wright-Giemsa. No obvious morphological differences were seen between Wnt3A-treated cells and control cells in eight cell lines (data not shown).

Figure 2 shows the data from flow cytometric analysis of representative cells. Eight cell lines showed no discrete cell populations in the $CD34^+CD38^-$ fraction. TMD7 and Jurkat cells were positive for CD34. Wnt3A stimulation did not affect the intensity of CD34 expression in these cells. Wnt3A slightly reduced the intensity of CD38 expression in KOPT-K1 (data not shown), TMD7 and OCI/AML3 cells. In the remainder, Wnt3A did not affect CD38 expression.

Effect of Wnt3A on β-catenin protein. Figure 3 shows immunoblots from representative cells. Treatment with SB216763 reduced the amount of phosphorylated β-catenin and resulted in accumulation of β-catenin in both cell lines, as expected. In DND-41 cells, Wnt3A stimulation also reduced the phosphorylated β-catenin and increased β-catenin, while Wnt3A had no obvious effect on β-catenin in TMD7 cells. Cleaved Notch1 fragment was not affected by either Wnt3A or SB216763 in either cell line.

Effect of Wnt3A on gene expression. We quantified *NOTCH1* and *JAGGED1* mRNA in cells with or without Wnt3A stimulation. The expression was normalized by the β-actin mRNA level, which was measured concurrently. There were no significant differences in *NOTCH1* and *JAGGED1* mRNA between the cells with and without Wnt3A in the eight cell lines (data not shown).

Discussion

In this study, we have shown that Wnt3A stimulation slightly suppressed the short-term growth in three out of eight cell lines, promoted the colony formation in one out of five cell lines and promoted the CCR in three out of five cell lines examined. These findings suggest that Wnt3A stimulation does not promote the proliferation of whole cell populations; rather it slightly suppresses it in some cell lines. However, Wnt3A stimulation promoted the amplification of clonogenic cells during suspension culture in some cell lines. Based on the results of primary colony assays, this promotion seems unlikely to be due to stimulation of colony formation, but rather due to an increase in leukemic stem or progenitor cells, which have the ability to form colonies.

We have verified that the recombinant Wnt3A protein used here activated the Wnt/β-catenin pathway by showing that stimulation reduced phosphorylated β-catenin and increased β-catenin. In some cells, such as TMD7 cells, Wnt3A stimulation caused neither a cellular response nor a change in β-catenin protein. These cells may not possess receptors able to respond to Wnt3A. There are many kinds of Wnt proteins and we cannot therefore rule out the possibility that other Wnt proteins are involved in the growth of these cells.

We tried to detect the increase in leukemic stem cells using flow cytometric analysis. However, discrete $CD34^+CD38^-$ cell populations, which are thought to be the fraction containing the leukemic stem cells (14), could not be detected in the cell lines examined. We found that Wnt3A stimulation slightly reduced the intensity of CD38 expression in three cell lines. This reduction may suggest a shift to an immature state, since CD38 is regarded as a differentiation marker. Its physiological significance is unclear, as the reduction was seen even in TMD7 cells, which showed no response in the culture assay and immunoblot analysis, as shown above.

It has been reported that Wnt and Notch signaling have a mutual relationship in the regulation of hematopoietic stem cells (1, 15, 17). In this study, Wnt3A stimulation neither up-regulated *NOTCH1* or *JAGGED1* mRNA nor activated Notch1 protein. The relationship between Wnt and Notch in leukemia cells might be different from that in normal hematopoietic cells.

In conclusion, we have shown that Wnt3A stimulation promoted the self-renewal capacity of leukemia stem/progenitor cells in some cell lines. The fact that Wnt signaling works not on differentiated cells, but on stem cells in normal hematopoiesis seems also to be applicable to leukemia cells. The elucidation of Wnt signaling will help in a better understanding of leukemic stem cells and will potentially lead to the development of new therapies targeting Wnt-related molecules in leukemic stem cells.

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