Promotion of the Self-renewal Capacity of Human Leukemia Cells by Sonic Hedgehog Protein

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Abstract. *Background: Hedgehog (Hh) signaling is involved in cancer cell growth. However, the effects of Hh stimulation on leukemia cells are unknown. Materials and Methods: The effects of recombinant sonic Hedgehog (Shh) protein on the in vitro growth of one B-lymphoma and four myeloid leukemia cell lines were examined. Results: Shh stimulation had no significant effect on the short-term growth of whole cell populations in any of the five cell lines. However, Shh promoted clonogenic cell recovery after suspension culture, suggesting promotion of leukemia stem or progenitor cell amplification in three cell lines. The lack of Hh receptors in one cell line and endogenous Shh expression in another were possible reasons for the lack of effects of Shh in these cases. Conclusion: These results suggest that Shh stimulation promotes the self-renewal capacity of leukemia stem cells in some cell lines. Inhibition of Hh signaling could represent a novel therapeutic approach in leukemia.*

Hedgehog (Hh) signaling plays an important role as an organizer in embryonic development, as well as in regulating hematopoietic stem cells (1), and in the growth of various types of cancer cells (2), such as glioma (3), breast cancer (4), and leukemia (5, 6). The Hh signaling cascade is as follows: in the absence of Hh ligands, the activity of Smoothened (Smo) is suppressed by Patched-1 (Ptch-1), which is an Hh ligand receptor. Ligand binding to Ptch-1 releases Smo, which then activates the Glioma-associated oncogene homolog (Gli) transcription factor, which in turn induces the transcription of various target genes. Hh comprises a family of three proteins: Sonic Hh (Shh), Indian Hh and Desert Hh, of which Shh is thought to play an important role in hematopoiesis (1).

Shh and Gli1 proteins were recently reported to be expressed in cells of various hematologic malignancies, especially in acute myeloid leukemia (AML) cells (5). Hh activation through the up-regulation of Smo is also reportedly required to maintain chronic myeloid leukemia stem cells (7, 8). We previously reported that the steroidal alkaloid cyclopamine, which blocks Hh signaling, suppressed the growth of some leukemia and lymphoma cell lines (9).

In this study, we examined the effects of recombinant Shh protein on the growth of AML and B-lymphoma cell lines in culture. We focused on its effects on the self-renewal capacity of leukemia stem cells by examining the amplification of clonogenic cells after suspension culture with Shh. To our knowledge, this is the first reported study showing the effects of Shh on the growth of leukemia cells.

Materials and Methods

Cells and reagents. Four human AML cell lines were used: OCI/AML1, OCI/AML5 (established at the Ontario Cancer Institute, Canada), HL-60 (supplied by the Japanese Cancer Research Resources Bank), and NB4 (kindly provided by Dr. M. Lanotte) (10); and one B-lymphoma cell line, TMD8, established at our laboratory from cells of a patient with leukemic stage diffuse large B-cell lymphoma (11). OCI/AML1 cell growth is dependent on granulocyte colony-stimulating factor (G-CSF). The growth of OCI/AML5 is dependent on G-CSF or granulocyte-macrophage colony-stimulating factor. The other three cell lines are growth factor-independent. Recombinant mouse Shh was purchased from R & D Systems (Minneapolis, MN, USA).

Short-term growth assay. The effects of Shh on short-term growth were examined using a colorimetric assay (WST-1 assay). Cells ($0.2–1\times10^4$ cells/well) were cultured in 0.1 ml of 2% fetal calf serum-supplemented RPMI-1640 medium (Gibco, Invitrogen, USA) with increasing concentrations of Shh (0-200 ng/ml). A solution containing WST-1 and 1-methoxy-5-methylphenazinium methylsulfate (Dojindo Laboratories, Japan) was added after 3-5 days. The optical density was measured after incubation using an enzyme-linked immunosorbent

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

Cell line	Colony assay		Suspension culture followed by colony assay					
	Mean colony no.		Cell no. ($\times 10^5$)		PE_s ($\times 10^{-2}$)		CCR ($\times 10^4$)	
	Control	Shh	Control	Shh	Control	Shh	Control	Shh
HL-60	394	414	11.0	9.3	11.6	23.2*	12.8	21.6*
NB4	428	418	4.5	4.2	6.1	17.8*	2.8	7.4*
TMD8	138	169	4.5	4.2	10.6	14.4	4.8	6.0
OCI/AML1	414	374	5.0	4.4	35.4	48.1	17.7	21.0
OCI/AML5	493	490	3.5	3.5	13.9	28.7*	4.9	10.1*

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

assay reader to provide an indication of cell number. Student's *t*-test was used to determine the statistical significance of differences between controls and treated cells.

Colony assay. The effects of Shh on colony formation by leukemia cells were examined in methylcellulose culture. Cells were plated at $0.2–1\times10^3$ cells/well in 96-well culture plates, in 0.1 ml of RPMI-1640 medium containing 0.8% methylcellulose, with or without Shh (200 ng/ml). After 7 days' culture, colonies containing more than 20 cells were counted under an inverted microscope.

Self-renewal capacity. The self-renewal capacity of leukemia cells was evaluated by the recovery of clonogenic cells after suspension culture, as described elsewhere (12). Using 24-well culture plates, 1×10^5 cells were cultured in 1 ml of RPMI-1640 medium with or without Shh (200 ng/ml). Cells were harvested after 3 or 4 days, and the cell numbers were counted. The harvested cells were then plated on 0.1 ml of methylcellulose-containing RPMI-1640 medium without Shh and colony numbers were counted after 7 days' culture. The plating efficiency following suspension culture (PEs) was evaluated as the mean colony number/number of cells plated. 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 Shh on differentiation, cells cultured with Shh as above were observed under an inverted microscope. Cytospin preparations were created from harvested cells and stained with Wright-Giemsa.

Reverse transcription-polymerase chain reaction (RT-PCR). The effects of exogenous Shh stimulation on the expression of *PTCH1, SMO,* and *SHH* genes were examined using RT-PCR. Cells were cultured with or without 200 ng/ml Shh for 24 hours. Total RNA was extracted and first-strand cDNA was synthesized. PCR was performed using primer sets (Search LC, Germany), according to the manufacturer's protocols. PCR products were electrophoresed on an agarose gel, and stained with ethidium bromide.

Results

Effects of Shh on short-term cell growth. Dose response curves showing the effects of Shh on the short-term growth of leukemia cells are shown in Figure 1. Shh stimulation tended to slightly suppress cell growth in all five cell lines tested, although the decrease was not significant. Each assay was repeated three times independently to verify their reproducibility.

Effects of Shh on colony formation and self-renewal capacity. The effects of Shh on colony formation are shown in the left-hand column in Table I. Shh stimulation had no significant effect on colony formation in any of the five cell lines. The right-hand column in Table I shows CCR as an indicator of self-renewal capacity. CCR was promoted by Shh stimulation in HL-60, NB4 and OCI/AML5 cells, but there was no significant effect of Shh in TMD8 and OCI/AML1 cells.

Effects of Shh on morphology. No obvious morphologic differences between Shh-treated and control cells were detected in cytospin preparation stained with Wright-Giemsa for any of the five cell lines (data not shown).

Effects of Shh on gene expression. The results of RT-PCR analysis are shown in Figure 2. HL-60, NB4 and TMD8 cells expressed *SHH* mRNA, and this expression was reduced by exogenous Shh stimulation. No *SHH* mRNA was detected in OCI/AML1 and OCI/AML5 cells. No expression of *PTCH1*, which is the Hh receptor gene, was detected in OCI/AML1 cells. Expression of *SMO*, which is the gene for an essential component of the Hh pathway, was detected in all five cell lines, and was reduced by Shh stimulation in HL60 cells.

Discussion

The results of this study demonstrated that Shh stimulation had no significant effect on short-term growth or colony formation in any of the leukemia or lymphoma cell lines examined, but did promote the amplification of clonogenic cells in three cell lines. This suggests that Shh does not affect

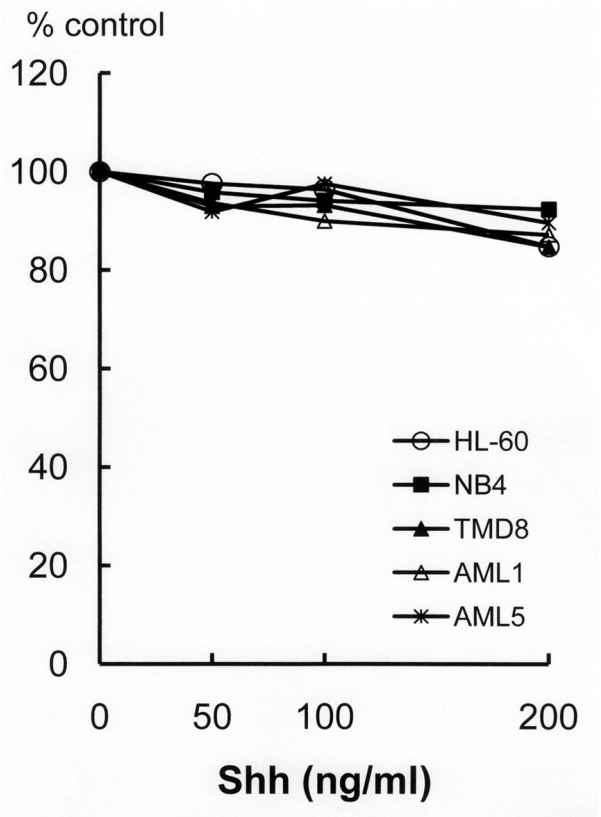

Figure 1. *Dose-response curves showing the effects of Shh on the growth of leukemia and lymphoma cell lines by WST-1 assay. Cells were cultured with increasing concentrations of Shh. Optical density (OD) was measured after 3-5 days. Growth is shown as a percentage of the mean OD value of control cells cultured without Shh.*

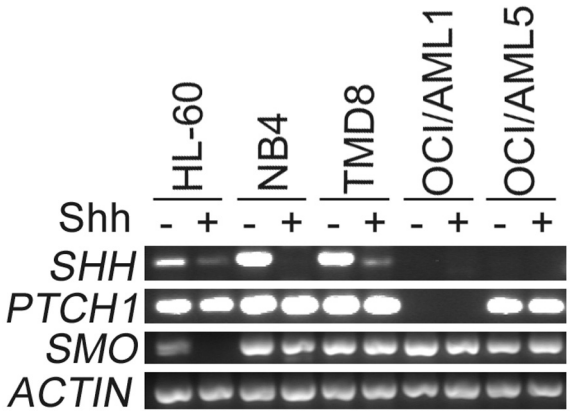

Figure 2. *RT-PCR analysis of Hedgehog-related gene expression. RNA was extracted from cells before and after culture with 200 ng/ml of Shh for 24 hours. cDNA was synthesized and PCR was performed. The expression of β-actin (ACTIN) was used as an internal standard. PCR products were electrophoresed and stained with ethidium bromide.*

the growth of the whole leukemia cell population, but does influence the self-renewal capacity of leukemia stem cells or the amplification of leukemia progenitor cells with the ability to form colonies. The failure of Shh to increase CCR in the remaining two cell lines can be explained as follows: OCI/AML1 cells do not express the Hh receptor, PTCH-1, as confirmed by immunoblot analysis (data not shown); TMD8 cells express endogenous SHH, which may be sufficient for Hh signaling, with no added effect of exogenous Shh on CCR.

In order to identify the mechanism whereby Shh stimulation promotes CCR, we examined its effects on the expression of genes for various factors related to growth and the cell cycle, such as signal transducer and activator of transcription 3 (*STAT3*), cyclin D1 (*CCND1*), p21 and hairy and enhancer of split 1 (*HES1*). However, Shh stimulation had no significant effect on the expression of these genes (data not shown), and we were therefore unable to elucidate the mechanism. Interestingly, the expression levels of *SHH* in HL-60, NB4 and TMD8 cells and the expression of *SMO* in HL-60 cells were reduced by Shh stimulation, which may have been the result of a negative feedback response, although the precise meaning of this is unknown.

Overall, the results of this study demonstrated that Shh stimulation promoted the self-renewal capacity or amplification of leukemia stem/progenitor cells in some leukemia cell lines. The elucidation of Hh signaling will help to provide a better understanding of leukemia stem cells and thus lead to the development of new therapies targeting Hh signaling molecules in leukemia stem cells.

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References

1 Bhardwaj G, Murdoch B, Wu D, Baker DP, Williams KP, Chadwick K, Ling LE, Karanu FN and Bhatia M: Sonic hedgehog induces the proliferation of primitive human hematopoietic cells *via* BMP regulation. Nat Immunol *2*: 142-143, 2001.

2 Yang L, Xie G, Fan Q and Xie J: Activation of the hedgehog-signaling pathway in human cancer and the clinical implication. Oncogene *29*: 469-481, 2010.

3 Ehtesham M, Sarangi A, Valadez JG, Chanthaphaychith S, Becher MW, Abel TW, Thompson RC and Cooper MK: Ligand-dependent activation of the hedgehog pathway in glioma progenitor cells. Oncogene *26*: 5752-5761, 2007.

4 Koga K, Nakamura Mk Nakashima H, Akiyoshi T, Kubo M, Sato N, Kuroki S, Nomura M, Tanaka M and Katano M: Novel link between estrogen receptor a and hedgehog pathway. Anticancer Res *28*: 731-740, 2008.

5 Bai LY, Chiu CF, Lin CW, Hsu NY, Lin CL, Lo WJ and Kao MC: Differential expression of sonic hedgehog and Gli1 in hematological malignancies. Leukemia *22*: 226-228, 2008.

6 Kobune M, Takimoto R, Murase K, Iyama S, Sato T, Kikuchi S, Kawano Y, Miyanishi K, Sato Y, Niitsu Y and Kato J: Drug resistance is dramatically restored by hedgehog inhibitors in CD34+ leukemic cells. Cancer Sci *100*: 948-955, 2009.
7 Dierks C, Beigi R, Guo GR, Zirlik K, Stegert MR, Manley P, Trussell C, Shmitt-Graeff A, Landwerlin K, Veelken H and Warmuth M: Expansion of Bcr-Abl-positive leukemia stem cells is dependent on hedgehog pathway activation. Cancer Cell *14*: 238-249, 2008.
8 Zhao C, Chen A, Jamieson CH, Fereshteh M, Abrahamsson A, Blum J, Kwon HY, Kim J, Chute JP, Rizzieri D, Munchhof M, VanArsdale T, Beachy PA and Reya T: Hedgehog signaling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature *458*: 776-779, 2009.
9 Kawahara T, Kawaguchi-Ihara N, Okuhashi Y, Itoh M, Nara N and Tohda S: Cyclopamine and quercetin suppress the growth of leukemia and lymphoma cells. Anticancer Res *29*: 4629-4632, 2009.
10 Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F and Berger R: NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood *77*: 1080-1086, 1991.
11 Tohda S, Sato T, Kogoshi H, Fu L, Sakano S and Nara N: Establishment of a novel B-cell lymphoma cell line with suppressed growth by gamma-secretase inhibitors. Leuk Res *30*: 1385-1390, 2006.
12 Tohda S, Yang GS, Ashman LK, McCulloch EA and Minden MD: Relationship between c-KIT expression and proliferation in acute myeloblastic leukemia cell lines. J Cell Physiol *154*: 410-418, 1993.

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