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 (G-CSF). The growth of OCI/AML5 is dependent on G-CSF or granulocyte 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 (0.2–1×10⁴ 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
Colony assay. The effects of Shh on colony formation by leukemia cells were examined in methylcellulose culture. Cells were plated at 0.2–1×10³ 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, 1×10⁵ 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 PEs.

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.
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


