Bone marrow stromal-cell line HS-5 affects apoptosis of acute myeloid leukemia cells HL-60 through GLI1 activation.

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Abstract

The Hedgehog (HH) pathway regulates cell proliferation and survival and contributes to tumorigenesis. However, not much is known about the role of the HH-signaling system in the bone marrow microenvironment during the development of Acute Myeloid Leukemia (AML). The aim of this study was to investigate the anti-leukemic effects of the HH-signaling pathway and molecular mechanisms of apoptosis induction in AML cells, by blocking the hedgehog signaling pathway during co-culture of AML cells with Bone-Marrow Stromal Cells (BMSCs). It was found that the HS-5 line of BMSCs protected AML cells from spontaneous apoptosis *via* up-regulation of transcription factors GLI1, BCL-2 and BCL-XL. These results indicate that the up-regulation of the transcription factors involved activation of the hedgehog pathway. Thus, targeting a microenvironment-related signaling pathway may be a novel approach to AML therapy.

Introduction

It has been clearly recognized that the hematopoietic microenvironment plays an important role in the pathogenesis of AML [1]. The bone marrow microenvironment, acting as a "tumor sanctuary", inhibits differentiation of leukemia and spontaneous or cytotoxic drug-induced apoptosis; and promotes migration and invasion. It can also lead to minimal residual disease, thereby increasing the probability of development of acquired drug resistance [2].

Hedgehog (HH) proteins were first identified in Drosophila as secreted signaling proteins important for embryonic development, tissue patterning, differentiation, organogenesis, tissue repair, and the maintenance of stem cells in adult tissues. Inappropriate activation of the HH signaling pathway occurs in several human cancers, including AML. In AML, HH signaling promotes cellular proliferation and survival [3], and enhances the self-renewing capacity of leukemic stem cells [4]. In addition, HH signaling promotes tolerance and resistance to chemotherapeutic agents. Recent studies suggest that stromally (fibroblast-) produced HH proteins-Indian (Ihh), sonic (Shh), and desert (Dhh) play some roles in the proliferation of hematopoietic stem cells and lymphoid cells. Stromally-induced hedgehog signaling may provide an important survival signal for B-cell and plasma-cell malignancies, B-cell Chronic Lymphocytic Leukemia (CLL) and diffuse, large B-cell lymphoma [5].

Small-molecule inhibitors of the Ihh signal transducer SMO have been studied in preclinical models, and have been applied in the treatment of various types of human cancers. Cancers sensitive to SMO inhibitors include Chronic Myeloid Leukemia (CML), some CLLs, and diffuse, large B-cell lymphoma. In other cancer types such as AML and some CLLs, these agents have demonstrated limited clinical activity. However, GANT61, a specific inhibitor of the transcription factors Gli1 and Gli2, is known to mediate strong *in vitro* and *in vivo* anti-tumor effects in several murine xenograft models, including neuroblastoma, pancreatic, prostate, and hepatocellular cancers [6]. Several studies reported superior anti-cancer efficacy of GANT61 relative to the SMO inhibitor cyclopamine or GDC-0449 [7]. Thus, targeting the GLII genes, which are mechanistically downstream from SMO and constitute the core targets in HH-dependent gene regulation, may provide a significant advantage in eliminating HH signaling.

However, the influence of stromally-induced HH signaling on the survival of AML cells, and the feasibility of targeting GLII

as a novel treatment for AML, have not been studied. Therefore, the aim of the present study was to determine the influence of bone marrow–derived stromal cells on the survival of AML cells, and to investigate the expression and functional modulation of GLII in AML cells due to HH signaling.

Materials and Methods

Materials

CCK8 was purchased from Dojindo. Annexin V-FITC/PI was purchased from KeyGEN BioTECH, Jiangsu, China. Antibodies were purchased from Abcam (GLI1/ab92611). GANT61 was obtained from Selleck Chemicals. DMEM and RPMI 1640 media were purchased from HyClone Laboratories (GE Healthcare Life Sciences, Logan, UT, USA). Fetal calf serum (FCS) and trypsin were products of Gibco (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell cultures

Human HS-5 fibroblastic stromal cell line was obtained from ATCC (American Type Culture Collection, Manassas, USA). HS-5 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humid atmosphere containing 5% CO_2. Human AML cell line HL-60 was obtained from Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). HL-60 cells were maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humid atmosphere containing 5% CO_2.

Co-culture of HL-60 cells with HS-5 cells

When the HS-5 cells reached confluency, the cells were trypsinized, washed, and transferred at a density of 10^5 cells/ml to 6-well flat-bottomed plates for co-culture studies. HL-60 cells pretreated with or without GANT61 for 48 h (30 µmol/L) were adjusted to a concentration of 5×10^5 cells/ml in RPMI1640 medium containing 10% FCS. Subsequently, the cells were transferred to 6-well flat-bottomed plates with or without HS-5 cells.

Cell counting Kit-8 assay

HS-5 cells were seeded onto 96-well plates at a concentration of 4×10^3 cells/well. On the next day, HL-60 cells treated with or without GANT61 (30 µmol/L) were cultured at a density of 5×10^4 cells/well, with or without a confluent HS-5 layer, for 24 h, 48 h, or 72 h. Then 10 µL of cholecystokinin-8 was added to each well. The plates were incubated at 37°C for 2 h, and thereafter absorbance was read at 450 nm in a scanning multi-well spectrophotometer (Bio-Rad Model 550, CA, US).

Measurement cell apoptosis

Apoptosis was determined using a staining kit based on Annexin V and Propidium Iodide (PI) according to the manufacturer's instructions. In the flow cytometric analysis of AML cell lines in co-culture with HS-5, non-adherent cells

were first harvested and subsequently the adherent cells were washed with pre-cooled PBS. Adherent and non-adherent cells were pooled, washed twice with pre-cooled PBS, and then re-suspended in FITC buffer. Annexin V-FITC (10 µL) was then added to the adherent cells and the set-up was incubated at 4°C for 15 min. For the non-adherent cells, 5 µL of annexin V-FITC was added and incubated at 4°C for 5 min.

RNA isolation and reverse transcription-PCR

Total RNA was extracted using TRIzol reagent. Complementary DNA was prepared by reverse-transcribing 2 µg of total RNA with reverse transcriptase reagents (Thermo Fisher Scientific Inc.). The specific primers for PCR amplification designed for this study are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal normalization control. Amplification programs were for GLI1: 30 cycles of: 95°C for 30 s; 57°C for 30 s, and 72°C for 45 s. For BCL-2: 30 cycles of: 95°C for 40 s; 60°C for 40 s and 72°C for 40 s. For BCL-XL: 30 cycles of: 95°C for 30 s; 59°C for 30 s; and 72°C for 30 s. The PCR products were analysed by electrophoresis in a 2% agarose gel. Relative expression was determined by densitometry using ImageJ software (Version 1.36b, NIH, Bethesda, Maryland). The mean values were normalized to the internal GAPDH control and were calculated from at least three independent experiments.

Table 1. *Primer sequences used for reverse transcription-PCR analysis.*

Gene	Primer sequences	Products (bp)
GLI1	F5'-TCCTACCAGAGTCCCAAGTTTC-3'	391
	R5'-CCAGAATAGCCACAAAGTCCAG-3'	
BCL-2	F5'-GAGGAGCTCTTCAGGGACGG-3'	151
	R5'-GGTGCCGGTTCAGGTACTCA-3'	
BCL-XL	F5'-ATGGCAGCAGTAAAGCAAGCG-3'	491
	R5'-TCATTTCCGACTGAAGAGTGA-3'	
GAPDH	F5'- ACCACAGTCCATGCCATCAC-3'	496
	R5'-TCCACCACCCTGTTGCTGTA-3'	

Immunofluorescence staining of GLI1

HL-60 cells were fixed in 4% formaldehyde, dropped on slides, air-dried, permeabilized, and blocked. The primary antibody used was rabbit anti-*GLI1*, and the secondary antibody was FITC/goat anti-rabbit (ZSGB-Bio, Beijing).The nucleus was counterstained using PI (Sigma Aldrich.). Immunofluorescent images were obtained on a BX/51 Olympus fluorescence microscope.

Statistical analysis

Experimental values are expressed as mean ± standard error of the mean unless otherwise indicated. Statistical significance

was analysed by using SPSS 19.0 software and was determined by one-way analysis of variance. P<0.05 was considered statistically significant. All results were produced from at least three independent experiments.

Results

Effects of a bone marrow stromal layer on the proliferation of HL-60 cells

HL-60 cells adhered to the HS-5 Bone-Marrow Stromal-Cell (BMSC) layer *via* pseudopodia. To determine the influence of HS-5 cells on the survival of HL-60 cells, the latter were cultured on HS-5 for 24, 48, 72, or 96 h, and the viability of the HL-60 cells was then determined by the Cholecystokinin-8 (CCK8) test. The spontaneous proliferation of HL-60 cells in normal culture media in the presence or absence of GANT61 was also tested, thereby excluding any direct influence of GANT61 treatment on survival or HH ligand expression in stromal cells. The HL-60 cells in co-culture group had a significantly higher rate of proliferation (p<0.05) than the HL-60 cells cultured alone. After treating HL-60 cells with GANT61, the proliferation of HL-60 cells in the co-cultured group was significantly inhibited (p<0.05).

HS-5 BMSCs reduced spontaneous apoptosis of HL-60 cells

To determine the influence of HS-5 cells on the survival of HL-60 cells, the cells were cultured on HS-5 BMSCs for 24, 48, or 72 h, and the apoptotic cell population was then determined by the annexin-PI assay. The results showed that HS-5 cells significantly decreased survival of HL-60 cells relative to HL-60 cells cultured in medium alone (p<0.05). These findings indicate that HS-5 BMSCs protected the HL-60 cells from spontaneous apoptosis. Treatment of HL-60 cells in a co-culture system with GANT61 induced apoptosis in HL-60 cells (p<0.05). These results show the positive influence of a stromal microenvironment on the survival of HL-60 cells, and further confirm the contribution of HH signaling to stromal-cell–induced HL-60 cell survival.

Co-culturing with HS-5 cells enhanced expressions of GLI1, BCL-2 and BCL-XL in HL-60 cells

Over-expression and activation of hedgehog signaling in the bone marrow niche involves crosstalk between AML cells and stromal cells. The results obtained from CCK8 and Annexin-PI assays were corroborated by results from RT-PCR for *GLI1*, *BCL-2* and *BCL-XL*, the target genes of HH signaling. There was an increased expression of *GLI1* in the presence of HS-5 cells, relative to when HS-5 cells were absent, which is consistent with the results of the CCK8 and annexin-PI assays. Together, these results clearly demonstrate that modulation of HH signaling in HL-60 cells influenced the survival of HL-60 cells, implying that HH signaling is involved in the pathogenesis of AML. Next, the expression of GLI1 protein in HL-60 cells was examined using immunofluorescence assay.

GLI1 expression was higher in the presence of HS-5 cells, when compared to GLII expression in its absence.

Discussion

The bone marrow microenvironment plays an important role in the initiation and progression of leukemia, and in the persistence of minimal residual disease and disease relapse. Inhibition of spontaneous apoptosis in leukemic cells by BMSCs, mediated by phosphorylation of the AKT/BCL-2 pathway, has a protective effect. Moreover, MS-5 stromal cells block apoptosis in HL-60 cells and in primary AML blasts *via* modulation of proteins in the BCL-2 family. Consistent with the above findings, the results of the present showed that HS-5 cells inhibited HL-60 cell apoptosis, and improved HL-60 cells survival. Emerging evidence suggest that HH signaling is aberrantly activated in various cancer types, including gastric [8], colorectal, prostate, and breast cancers. Therefore, targeting the HH signaling pathway may provide an effective therapeutic approach for the treatment of various cancers. Activation of the HH signaling pathway, which is often associated with stromal cells, has been reported in lymphoma and multiple myeloma, B-CLL; diffuse, large B-cell lymphoma, and myelodysplastic syndrome. Studies have shown that IHH and its signal transducer, SMO are expressed in CD34^{+} Acute Myeloid Leukemia (AML). Knockdown of HHIP in stromal cells increased their supporting activity although control cells marginally supported SMO[+] leukemic cell proliferation. The de-methylating agent, 5-aza-20-deoxycytidine restored HHIP expression *via* de-methylation of HHIP and reduced the leukemic cell-supporting activity of AML-derived stromal cells [9]. Modulation of HH signaling in B-CLL influenced the survival of B-CLL cells [10], indicating that HH signaling mediated by stromal cells promotes the survival of B-CLL cells.

In the present study, the effect of BMSC on the hedgehog signaling pathway was studied in HL-60 cells. The results showed that HS-5 BMSCs activated the hedgehog pathway in co-cultured HL-60 cells, thereby conferring survival advantage on the cells. The hedgehog signaling pathway inhibitor GANT61 was effective in causing cell death in HL-60 cells co-cultured with HS-5 cells. The results obtained in RT-PCR for *GLI1*, *BCL-2* and *BL-XL* were corroborated by results from Annexin-PI assays.

In summary, this study demonstrates that GANT61 inhibits HS-5-cell–induced hedgehog signaling in HL-60 cells. Activation of the hedgehog pathway in HL-60 cells is dependent on their interaction with stromal cells. These findings demonstrate a role of the tumor microenvironment in the survival of HL-60 cells, *via* stromal-cell–mediated HH signaling. Thus, the targeting of the microenvironment-related signaling pathway may be a novel therapeutic strategy for AML.

Funding

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