Effects of the Hedgehog Inhibitor GDC-0449, Alone or in Combination with Dasatinib, on BCR-ABL-Positive Leukemia Cells

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Hedgehog (Hh)-glioma-associated oncogene homolog (Gli) signaling is implicated in a large number of human cancers such as leukemia. In this study, we investigated the effects of the potent Hh antagonist GDC-0449 on the BCR-ABL-positive cell line OM9;22 and primary samples when leukemia cells were protected by a feeder cell line (S9 cells). The numbers of OM9;22 cells significantly increased with S9 cells. Treatment of OM9;22 cells with GDC-0449 caused cell growth inhibition and induced apoptosis. Moreover, GDC-0449 inhibited the colony growth of Philadelphia chromosome (Ph)-positive primary samples. We next investigated the effects of a combination of GDC-0449 and dasatinib on these cell lines. The growth inhibition typically promoted by dasatinib was significantly reduced in the presence of S9 cells. Treatment of Ph-positive leukemia cells with GDC-0449 and dasatinib in the presence of S9 caused significantly more cytotoxicity than that caused by each drug alone. Inhibition of Gli1 or Gli2 by siRNA transfection reduced the growth of the Ph-positive cell line K562 and increased cytotoxicity of dasatinib. Moreover, colony formations of Gli1 or Gli2 knockdown cells were also reduced. Data from this study suggest that administration of the Hh inhibitor GDC-0449 inhibits BCR-ABL-positive cell growth and enhances the cytotoxic effects of dasatinib in the presence of feeder cells.

Introduction

The chimeric protein BCR-ABL is the cause of chronic myeloid leukemia (CML) and Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia [1]. This oncoprotein carries a constitutively active form of the kinase domain. The deregulated BCR-ABL hyperactivates downstream signaling molecular pathways such as Ras, phosphatidylinositol-3 kinase (PI3-K)/v-akt murine thymoma viral oncogene homolog (Akt), and signal transducer and activator of transcription (STAT) 5 [2], thereby playing an important role in the pathogenesis of Ph-positive leukemia. PI3-K has been reported to be involved in imatinib resistance in Ph-positive cells [3].

The introduction of imatinib, the most successful molecular targeted therapy against CML, has revolutionized the treatment of patients with CML. Imatinib binds to the inactive conformation of the BCR-ABL protein and prevents its conformational switch to the active form. Imatinib inhibits the BCR-ABL pathway and induces apoptosis in CML cells. The new tyrosine kinase inhibitors (TKIs) dasatinib and nilotinib are effective in most patients with CML [4]. Despite such therapeutic interventions, CML is often incurable, largely because of the innate sensitivity of CML stem cells, particularly in their quiescent state [5]. Residual CML cells are present in niches of the bone marrow and are considered quiescent [6]. These leukemia stem cells are contained within a niche in the bone marrow and are often impervious to current treatments; thus, they maintain their proliferative activity when treatment ceases [7]. Therefore, new approaches against leukemia stem cells are required to treat Ph-positive leukemia patients.

The hedgehog (Hh)-signaling pathway identified in mammalian cells is a highly conserved signaling system that regulates the progenitor cells of proliferation and maintenance [8]. Three Hh ligands, namely Sonic Hh (Shh), Indian Hh, and Desert Hh, have been identified in humans. These Hh proteins are expressed in stromal cells of the bone marrow and are secreted as soluble ligands [9]. When Hh ligands bind to the PATCHED protein, which is a 12-pass transmembrane-spanning receptor, this complex represses the activity of smoothened (Smo) on target cells; therefore, if the inhibition of Smo is relieved, the Hh pathway will be activated. The downstream molecules of the glioma-associated oncogene homolog (Gli) protein play key roles in mediating the Hh-signaling pathway [9]. Although the Hh-signaling pathway plays an important role in tissue growth and repair, it is almost silent in adult tissues.

Recent studies have implicated the Hh pathway in the maintenance of stem cells in multiple myeloma and CML [10,11]. Therefore, Hh signaling has been recently shown to be
Biotechnology (Santa Cruz, CA). (sc-37913, sc-37913-V) were obtained from Santa Cruz siRNA or shRNA of Gli1 (sc-270268, sc-270268-V) and Gli2 (sc-28674, sc-28674-V). 

Statistical analysis

Student’s t test was used to determine if the effects of the drug treatments among the different groups were statistically significant.
significant compared to those in the control. Values of $P < 0.05$ were considered statistically significant.

**Results**

Enhanced OM9;22 cell proliferation in the presence of S9 cells and Shh

The bone marrow stromal cells create a permissive environment in which myelopoiesis and lymphopoiesis occur by the production of cytokines and growth factors. Therefore, in the first series of experiments, we analyzed the proliferation of the Ph-positive cell line OM9;22 and Ph-positive primary samples in the presence of feeder cells (S9). We found that the proliferation of OM9;22 and primary cells significantly increased in the presence of S9 cells compared to that in the absence of the S9 feeder cells (Fig. 1A, B). We next examined whether Shh was involved in OM9;22 cell proliferation. We found that compared to control cells, OM9;22 cell proliferation significantly increased in cells treated with 10 ng mL$^{-1}$ Shh for 24 h (Fig. 1C). We next investigated the efficacy of 100 nM dasatinib in the presence of Shh for 24 h. We found that the efficacy of dasatinib was reduced in the presence of Shh (Fig. 1C). In primary samples, we found that Shh was not involved in cell proliferation, but dasatinib activity was reduced in the presence of Shh (Fig. 1D).

**Efficacy of dasatinib on cell proliferation in the presence of feeder cells**

We next examined whether dasatinib activity was reduced in the presence of S9 cells. The number of OM9;22 cells decreased in a dose-dependent manner after treatment with

![FIG. 1. Ph-positive cell proliferation is enhanced in the presence of S9 cells. (A, B) OM9;22 and primary cells were cultured at a concentration of $2 \times 10^5$ mL$^{-1}$ in the presence or absence of feeder cells (S9) for 72 h. Viable cell numbers were calculated. Results are representative of 3 separate experiments. *$P<0.05$ compared to control. (C) OM9;22 cells were cultured at a concentration of $2 \times 10^5$ mL$^{-1}$ in the presence or absence of dasatinib and/or Shh for 24 h. Viable cell numbers were calculated. *$P<0.05$ compared to control or dasatinib treatment. (D) Primary cells were cultured at a concentration of $2 \times 10^5$ mL$^{-1}$ in the presence or absence of dasatinib and/or Shh for 24 h. Viable cell numbers were calculated. Ph, Philadelphia chromosome; shh, sonic hedgehog.
dasatinib for 72 h. Cell growth inhibition by dasatinib was significantly reduced in the presence of S9 cells (Fig. 2A). We next examined the levels of intracellular signaling molecules of the Hh pathway in S9 cells to determine which molecular pathways might be affected and produce this effect. Because dasatinib is a dual src/Abl kinase inhibitor, we expected dasatinib to inhibit the src kinase activity of the S9 cells. In this experiment, S9 cells were treated with dasatinib at a concentration of 100 nM for 24 h. In western blot analysis, src phosphorylation was decreased in response to dasatinib treatment in a dose-dependent manner. However, Shh levels were unaltered by dasatinib treatment. The Hh-Gli inhibitor

FIG. 2. Cell proliferation inhibition of OM9;22 by dasatinib is reduced in the presence of S9 cells. (A) OM9;22 cells (2 x 10^5 mL^{-1}) were cultured with dasatinib for 72 h in the presence or absence of S9. The cell growth percentage was examined. *P<0.05 compared to OM9;22 cells in the absence of S9. (B) S9 cells were treated with dasatinib or GDC-0449 for 24 h; total cellular lysates were analyzed with western blot using antiphospho (p)-src, src, Shh, and β-tubulin antibodies. Numerals indicate relative accumulation levels deduced by densitometry. (C) OM9;22 cells were treated with dasatinib for 24 h, and total extracts were examined by western blot analysis with anti-pAkt, Akt, pMAPK, Erk1, pCrk-L, Crk-L, pAbl, Abl, Gli1, Gli2, cleaved-PARP, and β-tubulin antibodies. Numerals indicate relative accumulation levels deduced by densitometry. PI3-K, phosphatidylinositol-3 kinase; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase.
GDC-0449 is reported to have a median maximal plasma level of 23.0 μM in a phase I study [12]. Thus, a 10–20 μM concentration of GDC-0449 was used in this study to examine the effects of GDC-0449 on S9 cells. The Shh levels in the S9 cells were decreased after GDC-0449 treatment in a dose-dependent manner (Fig. 2B).

**Intracellular signaling of OM9;22 cells in the presence of S9 cells**

Since OM9;22 cell proliferation was increased in the presence of feeder cells, we next examined the intracellular signaling of OM9;22 cells in the presence of the S9 feeder cells with or without dasatinib treatment. Compared to the controls, phosphorylation of Akt, MAPK, Crk-L, and Abl was increased in the presence of S9 cells. The downstream targets of Hh signaling, Gli1 and Gli2, were also increased. The activated form of PARP was found after dasatinib treatment; however, PARP activity was reduced in the presence of S9 cells (Fig. 2C).

**Efficacy of GDC-0449 on Ph-positive cell proliferation**

We next investigated the efficacy of GDC-0449 on Ph-positive cell proliferation. OM9;22 cells were treated with GDC-0449 at concentrations of 100 nM to 10 μM, and a cell proliferation analysis was performed. After 48-h treatment with GDC-0449, OM9;22 cell proliferation was inhibited in a dose-dependent manner (Fig. 3A). Another Hh inhibitor, SANT-2, was also effective against OM9;22 cell proliferation, and GDC-0449 was also found to inhibit OM9;22 cell proliferation in the presence of S9 cells (Fig. 3A). In western blot analysis, Crk-L, which is the downstream target of BCR-ABL, was not inhibited by the presence of GDC-0449. However, cleaved PARP was detected, as was apoptosis of OM9;22 cells after GDC-0449 treatment (Fig. 3B, C). We also evaluated the GDC-0449 efficacy using Ph-positive primary samples. After exposure to 10 μM GDC-0449, the percentage of colony formation was reduced in the treated cells compared to the control cells (Fig. 3D). The inhibition rate of colony numbers of primary cells was 40%–60%, suggesting that the activity of the Hh pathway is modulated in Ph-positive leukemia cells. We also found that expression of Gli1, which is the downstream molecule of the Hh pathway, was reduced after GDC-0449 treatment in OM9;22 and primary samples (Fig. 3E).

**Effects of co-treatment with GDC-0449 and dasatinib on Ph-positive cells**

We examined the efficacy of dasatinib and GDC-0449 combination treatment against OM9;22 cell proliferation. We found that the combination of GDC-0449 and dasatinib significantly enhanced antileukemic activity (Fig. 4A). We next examined this using primary samples. The clinical details of patients who provided samples are described in Fig. 4B. Mononuclear cells (2×10^5 mL^-1) were treated with dasatinib and/or GDC-0449 at the indicated concentrations. We found that GDC-0449 and dasatinib combination treatment inhibited primary sample’s cell growth (Fig. 4C). These results suggest that compared to each treatment alone, co-treatment with GDC-0449 and dasatinib has superior antileukemic activity against BCR-ABL-positive leukemia cells.

**Antiproliferative activity after siRNA and shRNA of Gli1 and Gli2 transfection**

To determine the effects of Gli1 and Gli2 in Ph-positive cells, we transfected the Gli1, Gli2, and control siRNA and shRNA into K562 cells. Immunoblot analysis revealed that Gli1 and Gli2 siRNA transfection caused >40% reduction of Gli1 and Gli2 protein levels after 48-h transfection. We then examined the intracellular signaling. In an earlier report, c-myc and cyclin D were downstream targets of Hh signaling [15]. The expression of c-myc and cyclin D was decreased after Gli1 and Gli2 siRNA transfection (Fig. 5A). The inhibition rate of c-myc and cyclin D was 30%–40%. The cell growth inhibition by dasatinib was significantly increased compared to the control siRNA transfectant cells (Fig. 5B). We found that Crk-L phosphorylation was not decreased; however, PARP activation by dasatinib was increased after cells were transfected with Gli1 and Gli2 siRNA (Fig. 5C). We next examined colony growth using the re-plating assay and found that the number of colonies was significantly reduced in the Gli1- and Gli2-shRNA-transfected K562 cells (Fig. 5D).

**Discussion**

In this study, we demonstrated that a Smo inhibitor, GDC-0449, induces cell growth inhibition in Ph-positive leukemia cells. Moreover, combined treatment with GDC-0449 and dasatinib significantly inhibited proliferation of the BCR-ABL-positive cell line OM9;22 and Ph-positive primary samples, respectively.

Recent studies suggest that the bone marrow niche is converted into an environment containing dominant signals that favor leukemia cell proliferation and growth [16]. Leukemia cell growth disrupts this normal bone marrow niche of hematopoietic progenitor cells and creates a tumor microenvironment [17]. Hematopoietic cells, including leukemia cells, are strictly regulated by bone marrow stromal cells. Intracellular signaling of leukemia cells is initiated by cellular adhesion and cytokine production by stromal cells [16]. Furthermore, Hh is secreted by bone marrow stromal cells and maintains progenitor cell populations.

The paracrine signaling in stromal cells might be critical to the Shh-mediated effects on Ph-positive leukemia cell progression. The present data suggest that Shh inhibits Ph-positive leukemia cell proliferation. Moreover, we demonstrated that the efficacy of dasatinib against Ph-positive cell proliferation decreased in the presence of Shh and feeder cells (S9). Furthermore, the Shh protein of S9 cells was not inhibited by dasatinib treatment, suggesting that Ph-positive leukemia cells were still supported by Shh in the presence of dasatinib. Several models denote the Hh pathway systems that exist in tumor environments [9]. Type I is ligand-independent, and type II is autocrine ligand-dependent, and type III is a paracrine or reverse paracrine signal system. Hh ligand is produced by stromal cells derived from the bone marrow, the spleen, or lymph nodes and maintains B-cell lymphoma and multiple myeloma cells [10,18]. Regarding the paracrine mechanistic
FIG. 3. GDC-0449 inhibits Ph-positive cell proliferation. (A) OM9;22 cells were cultured in the presence or absence of S9 and treated with GDC-0449 or SANT-2. Viable cell numbers were calculated. *P < 0.05 compared to control. (B) OM9;22 cells were treated with 20 μM GDC-0449 for 1–8 h. The cells were examined by western blot analysis. (C) OM9;22 cells were treated with 20 μM GDC-0449 for 24 h. The percentage of apoptotic cells was examined. *P < 0.05 compared to control. (D) Primary cells were treated with 10 μM of GDC-0449 for 14 days. A colony assay was performed. *P < 0.05 compared to control. (E) OM9;22 and primary cells were treated with GDC-0449 for 24 h, and total extracts were examined by western blot analysis with anti-Gli1 and β-actin antibodies.
FIG. 4. Co-treatment with GDC-0449 and dasatinib inhibits Ph-positive leukemia cell proliferation. (A) OM9;22 cells were cultured at a concentration of $2 \times 10^5$ mL$^{-1}$ in the presence or absence of GDC-0449 and/or dasatinib for 72 h. Percentage of cell growth was examined. * or ** signifies $P<0.05$ compared to dasatinib treatment. (B) The clinical details of patients were described. (C) Primary samples were treated with GDC-0449 and/or dasatinib for 72 h. Viable cell numbers were calculated. *$P<0.05$ compared to dasatinib treatment with S9.
FIG. 5. Antiproliferative activity after siRNA and shRNA of Gli1 and Gli2 transfection. (A) Gli1, Gli2, and control siRNA-transfected K562 cells were examined by western blot analysis with anti-Gli1, Gli2, c-myc, cyclin D, and β-tubulin antibodies.

(B, C) Gli1, Gli2, and control siRNA-transfected K562 cells in the presence or absence of dasatinib for 24 h. The percentage of cell growth and immunoblot was examined. *P < 0.05 compared to control siRNA-transfected cells.

(D) Gli1, Gli2, and control shRNA-transfected K562 cells were examined by a colony assay described in the Materials and Methods section. *P < 0.05 compared to control siRNA-transfected cells.
(type III) actions of Shh, our results are consistent with previous findings that Shh paracrine signaling is required for Ph-positive cell proliferation.

Imatinib treatment significantly improves survival in patients with Ph-positive leukemia. However, ABL TKI does not alleviate Ph-positive leukemia. Cancer stem cells that propagate leukemia cells are resistant to ABL TKI. Bone marrow stromal cells also provide structural and physiological support for hematopoietic cells, including leukemia cells. Recent evidence demonstrates that aberrant reactivation of the Hh-signaling pathway contributes to tumor initiation and progression in various human malignancies, such as BCR-ABL-positive leukemia, and maintains cancer stem cells [11,19,20]. In particular, the loss of Smo, an essential component of the Hh pathway, impairs hematopoietic stem cell renewal and decreases CML induction by the BCR-ABL oncogene. Smo loss also depletes CML stem cells. The expression of constitutively active Smo increases the frequency of CML stem cells and accelerates CML development, suggesting that Hh signaling is involved in CML cell maintenance [11].

Hh signaling regulates the self-renewal and survival of cancer stem cells in different tumor types. Thus, targeted Hh pathway inhibition might have beneficial effects against tumors. In particular, an Hh-Gli inhibitor, that is, cyclopamine, restricts tumor growth and prevents recurrence without any side effects in mouse xenograft models [21]. GDC-0449 is reported to be a more potent and specific Smo inhibitor and restricts tumor growth and prevents recurrence without any side effects in mouse xenograft models [21]. GDC-0449 is also shown to inhibit Smo expression in CML stem cells. The expression of constitutively active Smo increases the frequency of CML stem cells and accelerates CML development, suggesting that Hh signaling is involved in CML cell maintenance [11].

Hh signaling regulates the self-renewal and survival of cancer stem cells in different tumor types. Thus, targeted Hh pathway inhibition might have beneficial effects against tumors. In particular, an Hh-Gli inhibitor, that is, cyclopamine, restricts tumor growth and prevents recurrence without any side effects in mouse xenograft models [21]. GDC-0449 is reported to be a more potent and specific Smo inhibitor and is currently used in human clinical trials. The optimal use of Hh pathway inhibitors such as GDC-0449, alone or in combination with dasatinib, has not been examined until now. In this study, we demonstrated that GDC-0449 is a novel agent that induces antileukemic responses by inhibiting components of the Hh pathway such as cyclin D and c-myc.

The combined administration of an Hh inhibitor and dasatinib effectively inhibits Ph-positive leukemia cell proliferation, which is supported by feeder cells. Moreover, we have shown that combining Hh inhibitor with Abl TKI is very effective in overcoming the drug resistance of Ph-positive leukemia cells.

Acknowledgments

This work was supported by a High-Tech Research Center Project for private universities—a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology (MEXT)—and by the University–Industry Joint Research Project for private universities—a matching fund subsidy from the MEXT. This work was also supported by Grants-in-Aid for Scientific Research from the MEXT.

Author Disclosure Statement

The authors declare no conflicts of interest.

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Received for publication January 18, 2012
Accepted after revision May 25, 2012
Prepublished on Liebert Instant Online May 29, 2012