Inhibition of the Sonic Hedgehog Pathway by Cyclopamine or GLI1 siRNA Reduces In Vivo Tumorigenesis of Human Medulloblastoma Cells Xenotransplanted to Immunodeficient Nude Mice

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Abstract

Medulloblastoma is an aggressive tumor; grade IV of the WHO classification that develops in the cerebellum, mostly linked to infancy and adolescence. It can be classified histologically and molecularly in different subtypes. Morphologically it can be divided into classical, desmoplastic/nodular, anaplastic, and large cell medulloblastoma. Molecularly, there are four groups: Wnt, Shh (sonic hedgehog), group 3 (mainly linked to MYC amplification), and group 4 (for unclassified tumors). Our work tries to prove the inhibition of the Shh pathway in two medulloblastoma cell lines: DAOY, a desmoplastic cerebellar medulloblastoma cell line of the Shh molecular group; and D283 Med, derived from a metastasis to peritoneum, possibly corresponding to an aggressive group 3 medulloblastoma. Cyclopamine, an inhibitor of the SMO oncogenic protein, and GLI1 siRNA were used as inhibitory agents of the Shh pathway in the two cell lines. For the in vivo assay, for each cell line, each experimental group consisted of 6 mice injected subcutaneously with control cells on the right flank, and cells treated with cyclopamine or GLI1 siRNA on the left flank. In the cell lines studied, cyclopamine showed a high inhibitory growth of subcutaneous tumors in the D283 Med and DAOY lines. The siRNA treatment, however, was only effective in the D283 Med cell line.

Keywords

Medulloblastoma, Sonic hedgehog, Cyclopamine, GLI1 siRNA, Xenotransplantation

Introduction

Medulloblastoma, a tumor of the cerebellum, is one of the most frequent pediatric tumors [1], usually appearing in infancy and adolescence, and very rarely in adults. Some genetic syndromes, like Gorling, Turcot, and Li-Fraumeni, predispose to the development of medulloblastoma [2]. Surgery, radiotherapy and chemotherapy are used against this tumor, but patients suffer devastating neurocognitive sequelae due to the aggressiveness of these treatments [3]. For this reason, understanding the

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molecular mechanisms of the pathogenesis of medulloblastoma and designing new approaches to treat this disease are important in biomedical research [1].

This embryonic tumor is classified in four histological subtypes according to the World Health Organization: classical, desmoplastic/nodular, anaplastic, and large cell medulloblastomas [4,5]. Moreover, four molecular subtypes have recently been introduced: Wnt, Shh (sonic hedgehog), group 3 (usually presenting MYC amplification), and group 4 (linked to isochromosome 17q, and mostly including those medulloblastomas not clearly belonging to other groups) [6].

The subgroup of Shh medulloblastomas are produced by alterations in the sonic hedgehog signaling pathway. In the absence of the Shh ligand, PTCH inhibits the function of SMO, thereby inhibiting GLI1 activation [7]. In the presence of the Shh ligand, PTCH eliminates its inhibitory effect on SMO, which will promote activation of GLI1, its translocation to the nucleus, and thereby stimulation of transcription of its target genes such as GLI1, BMI1, HHIP, and SUFU. Aberrant and altered signaling of the Shh pathway leads to certain types of cancers and tumors [8].

A possible therapeutic strategy for the treatment of medulloblastoma might be the use of inhibitors of the Shh pathway such as cyclopamine that directly inhibits SMO function. Another possibility would be an approach to knock-down GLI1 by siRNA [1]. We have used the two approaches in two different medulloblastoma cell lines: DAOY, a desmoplastic cerebellar medulloblastoma cell line of the Shh molecular group; and D283 Med, derived from a metastasis to peritoneum, possibly corresponding to an aggressive group 3 medulloblastoma.

Previous results from our group demonstrated that both cell lines express several genes related to the Shh pathway (SHH, PTCH, SMO, GLI1, GLI2, GLI3, MYCN, BMI1, HHIP and SUFU) at similar levels [9], determined by RT-PCR experiments. SHH level of expression was a bit smaller in D283 Med than in DAOY, although SUFU slightly showed the opposite results. MYCN expression was similar in both cell lines, but none of them presented MYCN gene amplification (ratio above 10) assayed by qPCR, although DAOY presented an amplification ratio of 6.4, and D283 Med a ratio below 3 (unpublished results). We treated both cell lines with cyclopamine to inhibit the Shh pathway [9]. PTCH and GLI relative expression decreased after cyclopamine treatment, more noticeable in DAOY (60% decrease) than in D283 Med cells (20% decay). Respect to cell survival, 20% DAOY cells and 50% D283 Med cells survived the cyclopamine treatment. Finally, an in vitro tumorigenicity test, based on a colony forming assay in soft agar showed 80% and 50% decay in the number of colonies in DAOY and D283 Med treated cell lines respectively, compared to controls. These results show that DAOY, a Shh medulloblastoma cell line responds better to treatment with cyclopamine than D283 Med, as DAOY treated cells presented a higher decrease in PTCH and GLI expression, a lower cell survival, and a higher decay in colony formation in soft agar.

Next, we decided to further explore the inhibition of the Shh pathway in the DAOY cell line with a double method of treatment: cyclopamine (to inhibit SMO function, upstream of GLI1), versus GLI1 siRNA (to inhibit GLI1 itself) [1]. Cyclopamine inhibited GLI1 expression in DAOY cells by 85%, and siRNA inhibited GLI by 95%. The ability to form 2D colonies on cell plates suffered a 70% reduction under both conditions of treatment, while the ability to form 3D colonies in soft agar was reduced in a higher way (90% reduction with cyclopamine, and 95% reduction with GLI1 siRNA treatment, respect to controls). Finally, a cell migration wound (or scratching) assay was performed in the DAOY cell line, and showed that cell migration was reduced under both treatments compared to controls. These results show that cyclopamine and GLI1 siRNA both are relevant possibilities to in vitro treat Shh medulloblastoma cells: the reduction in 3D colonies in soft agar was even bigger than the reduction in 2D colonies, which might indicate that our treatments can reduce the cancer stem cell compartment, as 3D colonies are representative of the initiating and cancer-maintaining cell subpopulations. Even more, we have demonstrated that inhibition of Shh pathway by cyclopamine reduces the CD133+/CD15+ cell compartment and the in vitro tumorigenic capability of neuroblastoma cells [10]. And that CD133+ cells from medulloblastoma and PNET cell lines are more resistant to cyclopamine inhibition of the Shh signaling pathway than CD133- cells [9], which is in agreement with the theory that cancer stem cells are resistant to chemotherapy.

The present work promotes the idea of an in vivo experiment to further investigate the possibility of inhibiting the Shh pathway by two methods in medulloblastoma cell lines. In our experience [1,9,10], both cyclopamine and GLI1 siRNA appropriately behave in vitro assays, inhibiting the Shh pathway and reducing the cancer stem cells compartment. We treated the DAOY and D283 Med cell lines with cyclopamine or GLI1 siRNA and xenotransplanted them to immunodeficient nude mice for testing the capability of the treatments to reduce in vivo tumorigenesis respect to controls.

Materials and Methods

Cell lines

Medulloblastoma cell lines DAOY and D283 Med, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were used. The lines were cultured...
using RPMI L-Glutamax medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA), 4% non-essential amino acids (Lonza, Verviers, Belgium), 1% penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA) and 0.1% amphotericin B (Gibco, Invitrogen, Carlsbad, CA, USA). All cells used were maintained under normoxia conditions at 37 °C with a humid 5% CO2 atmosphere. Subcultures were performed at 80% confluence using trypsin/EDTA (Gibco, Invitrogen, Carlsbad, CA, USA) after pre-washing with phosphate buffered saline (Gibco, Invitrogen, Carlsbad, CA, USA). Both cell lines had previously been studied for expression of regulators of the Shh pathway by RT-PCR (SHH, PTCH, SMO, GLI1, GLI2, GLI3, MYCN, BMI1, HHIP, and SUFU) [9].

Xenotransplantation to nude mice

For xenotransplantation of human medulloblastoma cells to immunodeficient athymic nude mice, initially, a pilot trial was conducted for testing the ability of the DAOY and D283 Med cell lines to generate subcutaneous tumors in nude mice. We wanted the xenotransplant to produce a tumor that could grow in a continued way and not in an excessively accelerated way along time. For each cell line, 2, 4, 8 and 10 million untreated cells were injected subcutaneously into the flanks of the mice, in order to decide the appropriate number of cells for the experiment to be done with the treated cells. Each cell number was injected twice, into two distinct mice. The pilot experiment allowed the choice of the following cell numbers for the treated cell xenograft experiment: 10 million cells for D283 Med, and 4 million cells for DAOY.

Results and Discussion

In the cell lines studied, cyclopamine showed a high inhibitory growth of subcutaneous tumors in the D283 Med and DAOY lines (p < 0.0001). The siRNA treatment, however, was only effective in the D283 Med cell line (p < 0.0001) (Figure 1). Our work consisted of an in vivo tumorigenesis assay, by xenotransplantation of human medulloblastoma cell lines previously treated with cyclopamine or with GLI1 siRNA (and those not treated as a control) to immunodeficient nude mice. The study was performed with DAOY and D283 Med lines. Cy-
Figure 1: Growth of in vivo xenotransplantation of human medulloblastoma cells to immunodeficient nude mice. A) Inhibition of tumor growth by treatment with cyclopamine in DAOY cells. B) Inhibition of tumor growth by treatment with cyclopamine in D283Med cells. C) Inhibition of tumor growth by GLI1 siRNA treatment in DAOY cells. D) Inhibition of tumor growth by GLI1 siRNA treatment in D283Med cells. CP: cyclopamine. *: P<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001, according to ANOVA and multiple comparison test of Sidak.

E) Representative pictures of mice with xenografts of DAOY or D283Med treated cells. The xenotransplantation of the right flank of the mouse corresponds to the control treatment, while those of the left flank correspond to the treatments with cyclopamine (CP) or siRNA against GLI1.
cyclopamine could effectively inhibit the tumor growth of DAOY and D283 Med lines alike. The D283 Med cell line showed greater inhibition of tumor growth after siRNA treatment than DAOY. It might probably be due to baseline GLI1 expression differences in each cell line. Taking into account that the DAOY line is prototypical of the Shh type of medulloblastomas, it is clear that cyclopamine treatment can be very effective against it. The D283 Med line is more similar, on the contrary, to medulloblastoma groups not well known, but more aggressive than the Shh and Wnt groups, the so-called group 3 (associated with over expression of MYC) and group 4 (which accumulates those medulloblastomas not well classified in the preceding groups). Moreover, DAOY, besides presenting canonical activation of the Shh pathway, could be associated with non-canonical activation of this pathway, producing an increase of GLI1 not necessarily due to an increase in SMO activity, but to other regulators of cell signaling, external to the Shh pathway, such as TGF beta, PI3K/Akt, KRAS, MYC, or WNT/beta-catenin. If D283 Med is an example of group 3 medulloblastoma, with over expression of MYC, it will also over express GLI1, but perhaps in lesser amounts than the DAOY cell line through the canonical Shh pathway or through both the canonical and the non-canonical pathways. In such a case, if D283 Med produced lower GLI1 levels than DAOY, it would be plausible to think that siRNA against GLI1 would have greater effectiveness in D283 Med than in DAOY, as it indeed appears in our results.

As a conclusion of our results of this work, and after accumulating the experience of in vitro treatments against Shh [1,9,10], we may say that inhibition of the Shh pathway seems to be more efficient if an approach to directly inhibit GLI1 is applied, such as GLI1 siRNA, at least in the experiments in vitro. Quite the contrary, xenotransplantation studies to nude mice demonstrate that cyclopamine reduces in vivo tumorigenesis in the two cell lines, while GLI1 siRNA is effective only against the D283 Med cell line, and not against DAOY, which might indicate that Shh dependent medulloblastomas (like DAOY) should better be treated in vivo by cyclopamine, an SMO inhibitor, instead of by GLI1 siRNA knock-down.

Apart from our data, one may have into account that several other SMO inhibitors are being studied. Cyclopamine, the first developed inhibitor of SMO, is a teratogen that produces holoprosencephaly and cyclopia. Even, it cannot be administered orally, and has several side effects, making it impossible as a candidate to be used in humans [12]. Vismodegib is the first FDA-approved SMO inhibitor [13], in 2012, for the treatment of basal cell carcinoma and has been subsequently studied against colon [14], pancreatic [15], and medulloblastoma tumors [16]. In patients suffering from resistance to treatment [17-20], sonidegib, the second inhibitor of SMO approved by the FDA in 2015 [21], is used [22-24]. Other possible inhibitors against SMO, subjected to research, and possibly used in resistance to vismodegib and sonidegib [25], are natural products such as chalcone 12 [26], analogs of vitamin D3 [27], or itraconazole, the new inhibitor that might act against mutated SMO [28]. The appropriate use of all these compounds constitutes a new research area that requires basic knowledge of the Shh pathway regulation.

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References


