1. Abstract

Accumulated experimental evidence indicates that Hedgehog (Hh) signaling regulates cell proliferation and specification in a variety of organs during embryonic development. However, abnormal activation of this pathway in postnatal tissues has been linked to a large number of human cancers. With respect to the liver, it is known that Hh signaling not only influences bipotential precursor cells capable of pancreas and liver development, but is also implicated in the pathogenesis of liver tumors such as hepatoblastoma, hepatocellular and cholangiocellular carcinoma, if aberrantly activated. Blockade of Hh signaling by several specific inhibitors has been proven to successfully inhibit tumor growth of various Hh-associated cancers in vitro and in preclinical mouse models, and recent clinical data suggest that the implementation of novel anticancer therapeutics based on Hh interference into commonly accepted regimens are within reach. Thus, it is highly probable that Hh targeted therapies could be used for the treatment of Hh-dependent liver cancers in the future.

2. Introduction

Hedgehog (Hh) signaling plays a crucial role in a variety of aspects of vertebrate development, including pattern formation, proliferation, and differentiation of numerous cell types. Hh was first described in a screen aimed at identifying genes essential for embryonic patterning in the fruitfly, Drosophila melanogaster (1). The association between the Hh pathway and cancer was initially depicted in a human cancer predisposition disease, the Gorlin-Goltz or nevoid basal cell carcinoma syndrome, by identifying germ-line mutations in the Hh receptor gene Patched (PTCH), which results in inappropriate activation of the Hh pathway thereby leading to the development of basal cell carcinoma and other neoplasms, comprising medulloblastoma and fetal myogenic tumors (2-4). Since then, a whole plethora of cancers has been associated with deregulated Hh signaling, including tumors of the liver, such as hepatoblastoma (HB), hepatocellular (HCC) and cholangiocellular carcinoma (CCC).

Therapy optimization studies for malignant liver childhood tumors run over the last decades by the German
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Figure 1. The Hedgehog signaling cascade. (A) In the absence of Hh ligands, the PTCH receptor represses the SMO co-receptor by exclusion from the cell membrane, which leads to sequential phosphorylation of GLI by PKA, GSK3β, and CK1 and the proteolytic processing into amino-terminal GLI repressors. (B) Binding of the palmitoylated and cholesterol-modified biologically active amino-terminal form of HH to a complex of PTCH and CDO/BOC results in de-repression of SMO. SMO is then phosphorylated by GRK2 and translocated to the membrane. Hh ligands could also interact with HHIP to compensate for overactivity of Hh signaling in the physiological state. Activated SMO triggers release of SUFU from a microtubule-bound multi-protein complex consisting of FU, SUFU, KIF and GLI. This results in the stabilization of GLI, which then translocates to the nucleus initiating transcription of Hh target genes. Hh inhibitors (depicted in pink boxes) mainly act on the SMO co-receptor, but ligand/receptor interference and GLI perturbation also exist.

Since the initial discovery of Drosophila Hh (1), many components involved in Hh signal transduction have been identified and characterized. Although most components exhibit significant evolutionary conservation between flies and humans, important differences in specific signaling events have evolved in this pathway. However, we here focus on the current understanding of the vertebrate Hh network in order to discuss it’s relevance in development and treatment of liver cancers in humans.

3. THE HEDGEHOG SIGNALING CASCADE

The signaling pathway (Figure 1) is named after the secreted Hh ligands, which are encoded in mammals by the three genes sonic (SHH), desert (DHH), and indian hedgehog (IHH) (16). Hh proteins are synthesized as ~45 kDa precursors and cleaved to generate biologically active amino-terminal forms of 19 kDa. These signaling molecules are subsequently modified by covalently linking a palmitic acid moiety to the amino-terminus and a cholesterol group to the carboxy-terminal end (17, 18). In the absence of Hh ligands, the cell surface receptor patched (PTCH), a 12 pass transmembrane protein (19, 20), catalytically inhibits the activity of the 7 pass transmembrane receptor like protein smoothened (SMO), thereby keeping the signaling cascade in an off state (21). This leads to the sequential phosphorylation of GLI family proteins (5), the European (6), and the North American groups (7) have failed to show a substantial benefit for high risk patients with advanced, metastatic or recurrent disease. In addition, conventional chemotherapy had never proved to be effective for HCC in adults (8). Although the use of the tyrosine kinase inhibitor Sorafenib has recently shown some efficacy in HCC patients (9) and the implementation of this drug in a new HB therapy protocol is currently being discussed, serious efforts for developing new treatment strategies are essential. This review will focus on the findings established from recent studies on the role of Hh signaling in liver cancer and the possibility of using Hh targeted therapies for the treatment of this devastating disease. However, many important aspects of Hh biology are not discussed, but several excellent reviews have been published for those who are interested in more in-depth knowledge on the molecular mechanisms of the Hh pathway (10-13) and Hh inhibitors (14, 15).
transcription factors by protein kinase A (PKA), glycosgen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1) and proteolytic processing into amino-terminal GLI repressors (22, 23).

Binding of Hh ligands to PTCH leads to loss of the inhibitory activity of PTCH on SMO, which initiates an intracellular signaling cascade by recruiting downstream regulators (21). However, a couple of proteins at the cell surface are engaged to facilitate proper regulation of the initial activation process. The transmembrane proteins CDO (cell adhesion molecule-related/down-regulated by oncogenes) and BOC (brother of CDO) have been described to increase binding affinity of Hh to PTCH thereby positively contributing to Hh signaling (24). In contrast, all three Hh ligands could also interact with Hedgehog interacting protein (HHIP), an inhibitor of Hh signaling attached to the cell surface by a carboxy-terminal helix (25). Interestingly, a secreted version of HHIP has been described in the mature brain that is able to sequester Hh comparable to the membrane-associated form (26).

Upon Hh activation, SMO is phosphorylated by the G-protein coupled receptor kinase 2 (GRK2) and translocates to the primary cilia, an organelle of most vertebrate cells that extends into the extracellular environment (27, 28). Activated SMO acts on a microtubule-bound multi-protein complex consisting of fused (FU), suppressor of fused (SUFU), kinesins (KIF) and the zinc-finger transcription factor GLI. This leads to the release of the suppressive SUFU from the complex, stabilization of full-length GLI by FU, and nuclear localization of GLI to drive transcriptional activation of Hh target genes. In mammals, three different GLI factors (GLI1, GLI2 and GLI3) are known, with GLI1 being a strong transcriptional activator, GLI2 harboring both activator and repressor functions, and GLI3 with predominantly repressive effects (29). There is a still growing list of transcriptional targets that are activated by the GLI proteins comprising BCL2 (30, 31), FOXM1 (32), CCND1 (33), MYCN (34), IGFl (35) and PTCH itself (36), which leads to a negative feedback ensuring precise regulation of the pathway. In the physiological state of differentiated cells, the Hh signaling pathway is anticipated to be in the off state, which is consistent with the finding that adult mice largely tolerate inhibition of Hh signaling thereby qualifying Hh as an important factor during embryonic development (37).

4. HEDGEHOG SIGNALING IN LIVER DEVELOPMENT AND HOMEOSTASIS

Mammalian liver and ventral pancreas are believed to arise from a common progenitor within the ventral foregut endoderm (38). During the 2- to 6-somite stage of mouse embryogenesis (~E8.0 = 8 days of embryonic gestation) endodermal cells start to express Shh in response to fibroblast growth factor, which is secreted by the adjacent cardiac mesoderm (39). These Hh-responding endodermal cells subsequently form liver, whereas non-responding cells, which do not express Shh, form pancreas. At E9.0-9.5, Shh expression seems to become grossly lost in the hepatic endoderm, the region giving rise to the liver bud (40). However, the existence of single interspersed Hh activated cells in the fetal liver at E11.5 has been described by the use of Ptc1lacZ reporter mice, in which Hh activity can be reliably monitored by β-galactosidase staining in a highly sensitive manner (41). It became evident that Shh expression is most likely restricted to cells expressing Dlk1 (42), a marker for hepatoblasts (43). By using fluorescence-activated cell sorting Dlk1-positive hepatoblasts were the cells most strongly expressing Shh, whereas various other cell types contained in the fetal liver such as hematopoietic cells, erythroid cells, hepatic sinusoidal endothelial cells, and precursors of hepatic stellate cells/portal fibroblasts could be excluded to contribute to Shh expression. Interestingly, Hh signaling had an inhibitory effect on hepatic differentiation of Dlk1-positive hepatoblasts in culture, as evidence by decreased levels of the hepatocyte markers tyrosine aminotransferase and carbamoyl-phosphate synthetase 1 (42). Altogether, these studies strongly emphasize that Hh signaling is implicated in the regulation of cell fate decisions of bipotential cell populations in the endoderm thereby promoting specification of the early liver and moreover is maintained in selected cells during late-gestational embryogenesis to serve as a hepatic stem cell pool within the majority of Hh-quiescent mature epithelial liver cells.

Because the primary functional cells of the liver, hepatocytes and cholangiocytes (bile duct cells), grossly lack the expression of Hh-associated genes (44), the role of Hh signaling in postnatal and adult liver homeostasis has largely remained unnoticed. Surprisingly, Sicklick et al. (45) in the year 2006, published the first description of Hh-responsive hepatic progenitor cells in the adult liver (45). In this first paper in a row, the group of Anna Mae Diehl reported on the existence of small Hh activated cells in the periportal zone of the adult liver by the use of the Ptc1lacZ reporter mouse. Strikingly, most of these resident cells displayed a strong cytookeratin expression, a common marker for the committed, bipotential hepatic progenitor (45). Using EpCAM as a selection marker for hepatic progenitors, Hh components were highly enriched in freshly immunoselected human liver cells as compared to mature hepatocytes and non-epithelial liver cells. Moreover, when grown in culture these cells kept their Hh activity for weeks and were highly sensitive to the SMO antagonist cyclopamine, as evidenced by a substantial induction of apoptosis. Altogether, this initial study clearly indicated that the adult liver possesses a progenitor compartment that is regulated by the Hh pathway and might serve as a reservoir for life-long maintenance of tissue integrity. That this assumption holds true was established in various liver repair conditions provoked by alcohol consumption (46), fatty liver injury (47), and bile duct ligation (48).

5. HEDGEHOG SIGNALING IN LIVER CANCER

Since in most developmental systems and tissue repair responses Hh signaling is associated with enhanced growth and survival, it has been anticipated that overactivation of the pathway could be responsible for
proliferative diseases, including cancer. Hh signaling has been implicated in tumorigenesis following the discovery of germline mutations in the PTCH gene in patients with nevoid basal cell carcinoma syndrome (NBCCS), also named Gorlin-Goltz syndrome (3, 4). Patients with NBCCS are predisposed to basal cell carcinoma, medulloblastoma, and other tumors such as meningioma and rhabdomyosarcoma (2). Somatic PTCH mutations have subsequently been detected in sporadic cases of basal cell carcinoma, medulloblastoma and in rare cases of mammary tumors and meningiomas (49-53). The PTCH gene has since then been considered a classical tumor suppressor. However, there are several mechanisms by which the Hh pathway can be activated in human cancers, including SMO mutations in basal cell carcinoma (54) and medulloblastoma (55), SUFU mutations in medulloblastoma (56), as well as GLI1 amplification in rhabdomyosarcoma (57) and glioma (58). However, besides these mutation-associated malignancies various cancers have been described to be driven by either an autocrine or paracrine Hh stimulation caused by massive overabundance of Hh ligands secreted by the tumor cells themselves or the stromal surrounding. This second category of Hh-associated tumors comprise mainly small-cell lung cancer (59), pancreatic cancer (60), gastric cancer (44), prostate cancer (61, 62), and breast cancer (63). Interestingly, liver cancers both in children and adults have just recently been added to this list.

An important study by Berman et al. has shown that five out of nine cancer cell lines derived from the biliary tract show high autonomous Hh signaling activity (44). In line with this, the cholangiocellular carcinoma cell line HUCCT1 expressing high levels of the target gene PTCH could be effectively blocked by adding the SMO antagonist cyclopamine, thereby leading to a dramatic decrease in tumor cell viability (44). First studies on HCC emerged early in 2006, when Sicklick and colleagues as well as Patil and his coworkers simultaneously published their finding that HCC tumor samples and cell lines strongly express the ligands SHH and IHH as well as their downstream targets GLI1 and PTCH, suggesting an autocrine stimulation of the pathway for this tumor type (64, 65). A comprehensive study on 115 HCC specimens confirmed these first data by reporting on the consistent overexpression of GLI1 and PTCH in 52% of all cases, while adjacent normal liver tissue and stroma were negative for both genes (66). Interestingly, reduced expression of the HHIP gene, capable of competitively binding Hh ligands to compensate for overactivation of the pathway in some primary HCC (65) as well as a single missense SMO gene mutation in a 67-year-old female patient with a necrotic tumor and hepatitis C-induced cirrhosis (64) point to alternative activation mechanisms in HCC. However, all three studies clearly defined the Hh-dependency of HCC by inhibition of the Hh pathway using cyclopamine or a derivative thereof (KAAD-cyclopamine) in at least some HCC cell lines. As described for cholangiocellular carcinoma cells (44), blockade of the pathway resulted in a significant decrease of cell viability and proliferation. Consistent with earlier findings on other cancer cells with activated Hh signaling (67-69), cyclopamine induced apoptosis in HCC cells within a short period of treatment (65, 66). Of note, a fourth study could attribute a predominant role of GLI2 in HCC biology, since an antisense approach using oligonucleotides directed against GLI2 expression led to loss of growth and induction of apoptosis of HCC cells, whereas inhibition of GLI1 and GLI3 left most HCC lines unaffected (70).

HB representing the most common malignant liver tumor in children (71) was just recently added to the list of Hh-associated tumors (72). Our group has found high mRNA levels of the ligands SHH and IHH (72) as well as the downstream targets GLI1 and PTCH (Figures 2a and b) in a subset of primary HB and tumor cell lines, as compared to normal fetal and adult liver. Most interestingly, HHIP was barely detectable in HB cells (72), as already described for some HCC cell lines (65). Downregulation of HHIP could be ascribed to promoter hypermethylation in at least some cases, since CpG island hypermethylation was found in 6/23 HB and 1/1 transitional liver cell tumor, but none of the normal liver tissues (72). Strikingly, treatment of HB cells with the demethylating agent 5-aza-2’-deoxycytidine partially reverted the methylation status of the hypermethylated HHIP promoter region (Figure 2c) and re-established HHIP expression (Figure 2d) in at least some HB. Inactivation of other suppressive components of the Hh pathway such as SUFU and PTCH by promoter methylation was ruled out by bisulfite sequencing of the respective CpG islands. In line with the results in CCC and HCC, blocking Hh signaling by the SMO inhibitor cyclopamine also led to a significant decrease in cell viability and massive induction of apoptosis in HB cell lines (72).

Collectively, these studies clearly bolster the relevance of an activated Hh signaling pathway in liver tumors, both in children and adults. Although a comprehensive sequencing analysis of Hh components is lacking, autocrine stimulation through endogenous expression of Hh ligands in tumor cells along with transcriptional silencing of the HHIP gene by promoter hypermethylation can be anticipated as the driving forces for Hh activation in liver tumors.

6. HEDGEHOG TARGETED CANCER THERAPIES

Cancers harbor many mutations (73), but they appear to be dependent on derailment of only a few developmental pathways, including Hh signaling (74). Thus, manipulation of this pathway for therapeutic purposes has received increasing attention. The first inhibitor of Hh signaling becoming evident was cyclopamine, a steroidal alkaloid isolated from the corn lily, Veratrum californicum (75). Cyclopamine (Figure 1b) or synthetic derivatives thereof were first shown to block Hh signal transduction in tumors with PTCH and SMO mutations (76) and the exact targeting mechanism has been described almost two years later in the direct binding of cyclopamine to SMO (77). Treatment of tumor cells with cyclopamine has subsequently reported to induce decrease in proliferation, increase in apoptosis, and/or decrease in the metastatic behavior in a variety of cancers (44, 59, 60,
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Figure 2. Hedgehog dependency of hepatoblastoma. High expression of the Hh target genes GLI1 (A) and PTCH1 (B) in a large number of pediatric liver tumors as compared to normal fetal and adult liver suggests pathological activation of Hh signaling in this tumor type. Expression was measured by means of real-time RT-PCR and is depicted as relative RNA levels standardized against the house-keeping gene TATA-Box-binding-Protein. (C) Lack of HHIP expression in the Hh-activated hepatoblastoma cell line HepT3 is caused by heavy DNA methylation of the promoter region, as determined by bisulfite sequencing. Open and filled circles represent unmethylated and methylated CpG sites, respectively. Treatment of HepT3 cells with the demethylating agent 5-aza-2’-deoxycytidine results in demethylation of a large proportion of the HHIP promoter. (D) Demethylation of the HHIP promoter region after 5-aza-2’-deoxycytidine treatment for 72 h results in the reactivation of HHIP expression in HepT3 cells, as determined by real-time RT-PCR. For exact methodology, see (72).

As mentioned before, pathological activation of Hh signaling and its successful inhibition by cyclopamine has also been reported in cholangiocellular (44) and hepatocellular carcinoma (64-66, 70) as well as hepatoblastoma (72). These encouraging findings have fueled the race for identifying antagonists and small molecule inhibitors (69, 82-89), which more effectively block this deleterious pathway and overcome the poor aqueous solubility and acid lability of cyclopamine as well as its teratogenic effects (90).

One such inhibitor, CUR-61414 (Figure 1b) has been reported to be potent at nanomolar levels, suppresses proliferation and induces apoptosis in an in vitro basal cell carcinoma model consisting of Hh-induced basaioid nests derived from embryonic skin pouches of Ptc1+/− knockout mice (69). Another oral SMO inhibitor, HhAntag (Figure 1b) is also more potent than cyclopamine and completely eliminates growth of medulloblastoma in the Pch−/− knockout mouse (80). Interestingly, inhibitors upstream of SMO have also been described (Figure 1b). The Hh-neutralizing antibody 5E1 as well as the small-molecule robotnikinin both act on Hh signaling by disrupting binding of Hh ligands to PTCH (86, 91). Strikingly, anti-Hh 5E1 has already been effectively applied to block growth of Hh ligand-driven tumors in vivo (92). First approaches to develop inhibitors lying more distal of the pathway are also on their way (Figure 1b), which might hold promise to target tumors with initial mutations in SMO or an activated signaling cascade due to alterations of components downstream of SMO, such as SUFU and GLI. Theses include the HPI1-4 molecules known to inhibit SMO multimerization and trafficking as well as perturbation of GLI processing and stability (87) and GANT-61 and GANT-58 that attenuate DNA binding of GLI (83). Another potential avenue, especially with respect to liver tumors exhibiting lack of HHIP expression (65, 72), might be the creation of soluble decoy receptors that would titrate Hh ligands away from binding to the PTCH receptor. That truncated HHIP proteins are able to bind SHH and inhibit Hh signaling has recently been shown (93).
The most recently established inhibitor GDC-0449 (Figure 1b) was identified by Genentech in a high throughput screening of a library of small-molecule compounds and subsequently optimized through medicinal chemistry (94). As described for cyclopamine, GDC-0449 inhibits Hh signaling by specifically binding to SMO (95), but displays a greater potency and more favorable pharmaceutical properties. Besides being effective in a mouse model of medulloblastoma and xenograft models of colorectal and pancreatic cancer, GDC-0449 has already been successfully used in a phase I clinical trial on patients with locally advanced or metastatic basal cell carcinoma (94). Of the 33 patients treated, 18 had an objective response to GDC-0449, which was given in oral doses of 150 to 540 mg per day. Interestingly, no dose-limiting toxic effects or grade 5 adverse effects were observed in this two-stage trial, and only one grade 4 adverse event in terms of asymptomatic hyponatremia occurred during continuous daily administration for up to 19 months. Since expression analyses of archival tumor tissue of the phase 1 cohort showed increased transcriptional levels of GLI1 it was believed that the successful treatment of advanced basal cell carcinomas could be dependent on the inhibition of the activated Hh pathway (94). However, treatment of a single patient with metastatic medulloblastoma, which also depended on Hh pathway activation through a PTCH mutation, showed rapid regression of the tumor for a initial period of 2 months, but developed thereafter multiple new lesions and tumor regrowth at already existing sites (96). The cause for the resistance against GDC-0449 was identified in a mutation of the SMO gene giving rise to a protein with an altered topography at its carboxy-terminus, which is required for binding of cyclopamine (77). Nevertheless, these data provide the first proof of principle studies on the successful treatment of Hh-dependent tumors in patients. Altogether, Hh inhibitors are on their way to enter the clinic, and their ultimate utility, alone or in combination with conventional regimens, has to be determined in future clinical trials.

7. PERSPECTIVES

Considerable evidence has demonstrated that Hh signaling is deregulated in liver cancers and other neoplasias, and that pathologohal Hh activation drives survival and growth of cancer cells. Preclinical studies over the last decade making use of mouse models for a number of Hh-associated tumors have substantively shown that inhibition of Hh signaling could effectively block tumor growth and induce apoptosis. The success of the SMO inhibitor GDC-0449 in a clinical setting together with the finding that liver tumors both in children and adults belong to the ligand-driven group of Hh-activated cancers open the door to combination therapies for HB and HCC patients at advanced stages who cannot benefit from established treatments. Taken together, the data and studies summarized in this review strongly encourage the implementation of established SMO inhibitors into future therapeutic regimens of liver cancers.

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9. REFERENCES


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