Inhibition of Smoothened Signaling Prevents Ultraviolet B-Induced Basal Cell Carcinomas through Regulation of Fas Expression and Apoptosis

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ABSTRACT

Abnormal activation of the hedgehog-signaling pathway is the pivotal abnormality driving the growth of basal cell carcinomas (BCCs), the most common type of human cancer. Antagonists of this pathway such as cyclopamine may therefore be useful for treatment of basal cell carcinomas and other hedgehog-driven tumors. We report here that chronic oral administration of cyclopamine dramatically reduces (~66%) UVB-induced basal cell carcinoma formation in Ptch1+/− mice. Fas expression is low in human and murine basal cell carcinomas but is up-regulated in the presence of the smoothened (SMO) antagonist, cyclopamine, both in vitro in the mouse basal cell carcinoma cell line ASZ001 and in vivo after acute treatment of mice with basal cell carcinomas. This parallels an elevated rate of apoptosis. Conversely, expression of activated SMO in C3H10T1/2 cells inhibits Fas expression. Fas/Fas ligand interactions are necessary for cyclopamine-mediated apoptosis in these cells, a process involving caspase-8 activation. Our data provide strong evidence that cyclopamine and perhaps other SMO antagonists are potent in vivo inhibitors of UVB-induced basal cell carcinomas in Ptch1+/− mice and likely in humans because the majority of human basal cell carcinomas manifest mutations in PTCH1 and that a major mechanism of their inhibitory effect is through up-regulation of Fas, which augments apoptosis.

INTRODUCTION

The hedgehog (Hh) pathway plays a critical role in embryonic development and tissue polarity (1). Secreted Hh molecules bind to the receptor patched (PTC), thereby alleviating PTC-mediated suppression of smoothened (SMO), a putative seven-transmembrane protein. SMO signaling triggers a cascade of intracellular events, leading to activation of the pathway through GLI-dependent transcription (2). Considerable insight into the role of the Hh pathway in vertebrate development and human cancers has come from the discovery that mutations of the patched gene (PTCH1) underlie the basal cell nevus syndrome, a rare hereditary disorder in which patients are highly susceptible to the development of large numbers of basal cell carcinomas and other tumors (3, 4). Activation of Hh signaling, usually due to loss-of-function somatic mutations of PTCH1 and less often to activating mutations of SMO, is the pivotal abnormality in sporadic basal cell carcinomas (5–7). Therefore, targeted inhibition of SMO signaling should afford mechanistically based prevention/therapy of basal cell carcinomas, as well as of other tumors driven by Hh signaling abnormalities, including certain medulloblastomas, small-cell lung carcinomas, and gastrointestinal tract cancers (8–11). One such inhibitor is the naturally occurring plant extract cyclopamine, and there are additional synthetic compounds that directly associate with the transmembrane domains of SMO (12–14). Therefore, these small molecular weight compounds have significant promise for the prevention and treatment of basal cell carcinomas and other human malignancies.

Ptch1+/− mice (15) provided the first practical animal model for inducing basal cell carcinomas using UV and ionizing radiation (16). We report here that chronic oral administration of cyclopamine dramatically inhibits basal cell carcinoma growth in these mice. We also have tested the in vitro effects of cyclopamine and of the synthetic SMO inhibitor Cur61414 on the mouse basal cell carcinoma cell line ASZ001 and have demonstrated that both compounds elevate Fas expression and augment apoptosis. The clinical relevance of our data for treatment of basal cell carcinomas is supported by the low baseline Fas expression in basal cell carcinomas of both humans and mice and by the in vivo induction of high level Fas expression by short-term administration of cyclopamine in murine basal cell carcinomas. Thus, our studies support the idea that treatment of human basal cell carcinomas with specific inhibitors of the Hh pathway may offer a mechanism-driven approach to the chemoprevention of these tumors.

MATERIALS AND METHODS

Animals. Ptch1+/− heterozygous knockout mice have been developed by deleting exons 1 and 2 and inserting the LacZ gene at the deletion site (15). Ptch1−/−lacZ-transgenic mice were genotyped by PCR amplification of genomic DNA extracted from tail biopsies (15, 16). The animals were housed under standard conditions (fluorescent lighting 12 hours per day, room temperature 23°C to 25°C, and relative humidity 45 to 55%). The mice were provided tap water and Purina Laboratory Chow 5001 diet (Ralston-Purina Co., St. Louis, MO).

UV Light Source. An UV Irradiation unit (Daavin Co., Bryan, OH) equipped with an electronic controller to regulate dosage was routinely used for these studies. The UVB source consisted of eight FS72T12-UVB-HO lamps emitting UVB (290 to 320 nm, 75 to 80% of total energy) and UVA (320 to 380 nm, 20 to 25% of total energy). We used a Kodacel cellulose film (Kodacel TA401/407) to eliminate UVC radiation. A UVC sensor (Oriel’s Goldilux UVC Probe) was used during each exposure to confirm the lack of UVC lamps (290 to 320 nm, 20 to 30% of total energy) and the UVB source was calibrated using an IL1700 Research Radiometer/Photometer from International Light, Inc. (Newburyport, MA). The distance between the radiation source and targets was maintained at 30 cm. The irradiation assembly is kept in an air-conditioned room, and a fan is placed inside the exposure chamber to minimize temperature fluctuations during irradiation.

Carcinogenesis Protocol and Statistical Analyses. Mice were irradiated with a UV Irradiation unit (240 mJ/cm² three times a week) from age 6 to 32 weeks, at which time, ~50% of the animals had one or more visible skin tumors. The mice (25 mice per group) were given either cyclopamine (10 μg/day as a cyclohexanone complex) or the vehicle control in drinking water,
and the number of tumors was recorded weekly. Mice treated with cyclopamine or with the vehicle control were sacrificed at 52 weeks, their dorsal skin removed, and tumors harvested and collected for the histologic and immunohistochemical studies. The microscopic basal cell carcinoma areas were assessed by histologic evaluation of three dorsal skin sections per mouse from a total of seven mice (n = 7) in the vehicle-treated water group and a total of six mice (n = 6) in the cyclopamine-treated group. The basal cell carcinoma areas were measured by microscopic assessment using the Axiovision 3.1 analysis program (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Results were analyzed using the Student’s t test or a nonparametric test (Mann-Whitney test): P < 0.05 was considered statistically significant.

For evaluation of tissue expression and apoptosis in vivo, cyclopamine was injected (at 100 pg s.c. or intratumorally) into mice with visible basal cell carcinomas (>1 cm in diameter). Seventy-two hours later, basal cell carcinoma mass were embedded in OCT compound (Tissue-Tek; Sakura, Torrance, CA), and stored in −20°C for additional analyses. Stromal cells were used as the control for basal cell carcinoma cells.

β-Galactosidase Staining. Tissues were fixed in 0.2% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) or 2% formaldehyde (Fishier Scientific Co., Pittsburgh, PA) in 1× PBS for 20 minutes at 4°C, then washed twice in 1× PBS. Tissues were incubated with 5% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in 95% iron buffer solution for 24 hours at 37°C using a β-galactosidase staining set (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s guideline. The tissues were washed twice in 3% DMSO in 1× PBS and then three times in 70% ethanol. The tissues were embedded in paraffin and processed for counterstaining.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) and Immunofluorescent Staining. TUNEL analysis was performed using a kit from Roche Applied System according to the manufacturer’s guideline. Immunofluorescent staining of Fas in basal cell carcinomas was performed with an antibody specific to mouse Fas (M20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Cell Culture and Cell Viability Assay. The mouse basal cell carcinoma cell line ASZ001 was maintained in 154CF medium as reported previously (17). Ectopic expression of Gli1 in ASZ001 cells was induced using Lipofectamine 2000, and the transfected cells were cultured by selection after coexpression of green fluorescence protein and Gli1 (green fluorescence protein to Gli1 plasmid ratio = 1:4). C3H10T1/2 cells were cultured in basal medium containing 10% heat-inactivated fetal bovine serum. Gli1-transformed baby rat kidney cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum. GLI-transformed baby rat kidney cells were cultured in DMEM containing 10% fetal bovine serum. ASZ001 cells were first treated with 2×10^5 Ptch1+/−/− mouse. The Hh pathway is constitutively activated in essentially all human and mouse basal cell carcinomas (3, 20). Because PTCH1, which is frequently inactivated in human basal cell carcinomas, is known to be upstream of SMO (21), inhibiting SMO functions should be an effective way to treat basal cell carcinomas. To test the effects of a SMO antagonist on development of basal cell carcinomas, we administered cyclopamine orally to basal cell carcinoma-bearing Ptch1+/−/− mice. We UV-irradiated Pesth1+/−/− mice from age 6 to 32 weeks, at which time, approximately half of the mice had developed one or more macroscopic tumors: basal cell carcinomas, squamous cell carcinomas, and/or spindle cell tumors (fibrosarcomas). UV irradiation was then stopped and the mice were randomized (25 mice per group) to receive either vehicle or cyclopamine (as cyclohexanone complex) in the drinking water for the ensuing 20 weeks. The survival of these two groups of animals was similar, indicating that cyclopamine does not affect the overall survival of Pesth1+/−/− mice (data not shown). At age 52 weeks, we measured microscopic basal cell carcinoma areas per tissue area (mm²) in these two groups after β-galactosidase staining (see Materials and Methods for details) and found a 90% reduction of microscopic basal cell carcinomas in the cyclopamine-treated animals (Fig. 1, A and B). Cyclopamine-treated mice also had fewer visible basal cell carcinomas at age 52 weeks than did the vehicle-treated controls (Fig. 2, A and B). Thus, the mice receiving vehicle alone continued to develop visible tumors, and the number of tumors had tripled by week 52. We

RESULTS

Cyclopamine Inhibits Basal Cell Carcinoma Development in Pesth1+/−/− Mice. The Hh pathway is constitutively activated in essentially all human and mouse basal cell carcinomas (3, 20). Because PTCH1, which is frequently inactivated in human basal cell carcinomas, is known to be upstream of SMO (21), inhibiting SMO functions should be an effective way to treat basal cell carcinomas. To test the effects of a SMO antagonist on development of basal cell carcinomas, we administered cyclopamine orally to basal cell carcinoma-bearing Pesth1+/−/− mice. We UV-irradiated Pesth1+/−/− mice from age 6 to 32 weeks, at which time, approximately half of the mice had developed one or more macroscopic tumors: basal cell carcinomas, squamous cell carcinomas, and/or spindle cell tumors (fibrosarcomas). UV irradiation was then stopped and the mice were randomized (25 mice per group) to receive either vehicle or cyclopamine (as cyclohexanone complex) in the drinking water for the ensuing 20 weeks. The survival of these two groups of animals was similar, indicating that cyclopamine does not affect the overall survival of Pesth1+/−/− mice (data not shown). At age 52 weeks, we measured microscopic basal cell carcinoma areas per tissue area (mm²) in these two groups after β-galactosidase staining (see Materials and Methods for details) and found a 90% reduction of microscopic basal cell carcinomas in the cyclopamine-treated animals (Fig. 1, A and B). Cyclopamine-treated mice also had fewer visible basal cell carcinomas at age 52 weeks than did the vehicle-treated controls (Fig. 2, A and B). Thus, the mice receiving vehicle alone continued to develop visible tumors, and the number of tumors had tripled by week 52. We
We next assessed cyclopamine-induced apoptosis using flow cytometry analysis and found that treatment of cells with cyclopamine for 36 hours caused a 3-fold increase in the sub-G₁ cell population and a decrease in the S phase (Fig. 3C), suggesting that cyclopamine is a potent inducer of apoptosis in basal cell carcinomas. Treatment of ASZ001 cells with either cyclopamine or Cur61414 for 36 hours (see Materials and Methods for details) increased the level of activated caspase-3, a major executioner of cysteinyl aspartate-specific proteases for apoptosis (Fig. 3C).

Confirming our in vitro findings, direct injection of cyclopamine into basal cell carcinoma-bearing mice for 72 hours enhanced apoptosis (an increase in TUNEL-positive cells) of basal cell carcinoma tumor cells in vivo (Fig. 3D).

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Fig. 3. Cyclopamine inhibits the Hh pathway and induces apoptosis in ASZ001 cells. A. Gli1 and HIP transcripts were detected by real-time PCR analysis. Cells were treated with 2 µmol/L KAAD-cyclopamine for 12 hours. After RNA extraction, real-time PCR analysis was performed (see Materials and Methods for details). B. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of ASZ001 cells in the presence of SMO antagonists. ASZ001 cells were treated with cyclopamine, or the control compound tamotidine for 36 hours, and the cell viability was examined by MTT assay. C. cell cycle and protein analyses of ASZ001 cells. The percentage of cell population in each cell cycle.

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Active Fas/FasL Interactions Are Necessary for Cyclopamine-induced Cell Death. Because treatment of human basal cell carcinomas with IFN-α may be accompanied by increased Fas expression in the tumor (19, 22), we tested whether cyclopamine, too, can augment Fas expression. Indeed, cyclopamine substantially increased the level of Fas protein in ASZ001 cells (Fig. 4A). In contrast, we detected FasL protein irrespective of cyclopamine treatment (Fig. 4A). Using an ELISA assay, we detected FasL in the culture medium of ASZ001 cells, indicating that these basal cell carcinoma cells indeed secrete FasL protein (see Fig. 5B for details). Thus, Fas would appear to be the limiting factor for the FasL/Fas signaling axis in this basal cell carcinoma cell line. Conversely, Fas is down-regulated in C3H10T1/2 cells with stable expression of activated SMO via retrovirus-mediated gene transfer (Fig. 4B).

The in vivo relevance of the data obtained from the experiments with the ASZ001 cell line is supported by the low level baseline expression of Fas in human basal cell carcinomas (refs. 23, 24; Fig. 4C). The induction of Fas expression and apoptosis in Pch11/1/2 mouse basal cell carcinomas by treatment with either cyclopamine (Fig. 4D) or by Cur 61414 (data not shown). Specifically, Fas protein expression increased after cyclopamine injection (either s.c. injection or intratumoral injection), and this was accompanied by increased TUNEL-positive cells (Figs. 3F and 4D). Immunofluorescent staining revealed a membrane localization of Fas protein in basal cell carcinoma cells (indicated by the arrowhead in the insert of Fig. 4D). Thus, it appears that Fas expression is elevated in the presence of SMO antagonists both in cultured cell lines and in basal cell carcinomas induced in Pch11/1/2 mice.

On the basis of these results, we predicted that either (a) interruption of the FasL/Fas signaling axis or (b) inhibition of the downstream apoptosis-effector caspase 8 activity would prevent cyclopamine-induced apoptosis. To test this hypothesis, we inactivated FasL/Fas signaling using neutralizing antibodies against FasL. By depleting FasL molecules (Fig. 5B), the cyclopamine-mediated decrease in cell viability was rescued (Fig. 6, C and D). Neutralizing antibodies against FasL also decreased the level of caspase-3 (Fig. 5C). Furthermore, administration of the caspase-8 inhibitor Z-IETD-FMK (25) abrogated the cyclopamine-mediated activation of caspase-3 (Fig. 5C). Thus, our data provide direct evidence that the FasL/Fas signaling axis is an important mediator of cyclopamine-induced apoptosis in basal cell carcinomas.
The Molecular Basis of Cyclopamine-mediated Induction of Fas. To investigate the mechanism whereby inhibition of the Hh pathway induces Fas expression, we assessed whether this regulation occurs directly as opposed to indirectly by altering other signaling pathways. It has been reported that several signaling pathways, including the Ras-Erk pathway and the p53 pathway, can regulate Fas expression (26–28). We have previously reported that Hh activation augments Ras-Erk signaling (17). Consistent with those findings, we observed that cyclopamine decreased the levels of PDGFR-α and phospho-Erk, indicating an inhibitory effect on Ras-Erk signaling in ASZ001 cell (Fig. 6B). As a result, PDGF-A had no effect on cyclopamine-mediated caspase-3 activation, Fas induction, or cell death (see Fig. 6, D and E, for details). In contrast, epidermal growth factor, which can activate the Ras-Erk pathway through the epidermal growth factor receptor, did inhibit cyclopamine-mediated accumulation of the sub-G1 population (Fig. 6C), Fas expression (Fig. 6D), caspase-3 activation (Fig. 6D), and cell death (Fig. 6E). Furthermore, addition of the mitogen-activated protein kinase kinase inhibitor U0126 alone was sufficient to induce caspase-3 in ASZ001 cells (Fig. 6D). Consistent with these data, ASZ001 cells in which PDGFR-α and active Raf were overexpressed resisted cyclopamine treatment (data not shown), providing additional information that inhibition of PDGFR-α and subsequent down-regulation of Ras-Erk signaling is an important mechanism whereby cyclopamine induces apoptosis in basal cell carcinomas.

Our model predicts that overexpression of Gli1 in ASZ001 cells under a strong promoter (such as the cytomegalovirus promoter) would constitutively activate the Hh pathway, which could render these basal cell carcinoma cells resistant to cyclopamine treatment. Indeed, cyclopamine did not induce apoptosis in constitutive Gli1-expressing ASZ001 cells, as indicated by lack of TUNEL staining and of procaspase-3 cleavage (Fig. 6, F and G). As a result of constitutive Gli1 overexpression, PDGFR-α remained unchanged even after cyclopamine treatment (Fig. 6G). In addition, Fas protein was not induced by cyclopamine in constitutive Gli1-expressing ASZ001 cells (Fig. 6G). The ability of Gli-1 overexpression to abrogate cyclopamine-mediated cell death was additionally confirmed by flow cytometry analysis (data not shown). Although cyclopamine caused an increase in the sub-G1 population in Gli1-negative cells, no such change was observed in Gli1-expressing ASZ001 cells. These data indicate that ectopic expression of Gli1 under the cytomegalovirus promoter prevents cyclopamine-induced changes in the expression of PDGFR-α, Fas, and apoptosis.

DISCUSSION

The identification of SMO antagonists has created the opportunity to consider mechanism-driven anticancer strategies for effective treatment of common malignancies in which Hh activation is thought to be important, including basal cell carcinomas, as well as subsets of medulloblastomas, lung cancer, and gastrointestinal cancers (3, 8–11). Basal cell carcinomas frequently contain mutations of the PTCH1 gene, most of which lead to inactivated PTCH1 and consequently uncontrolled SMO signaling. Because PTCH1 is upstream of SMO (21),7 we reasoned that administration of the SMO inhibitor cyclopamine to Ptc1+/− mice could specifically and effectively inhibit the development of basal cell carcinomas in vivo. Our studies indicate that orally administered cyclopamine is a potent inhibitor of basal cell carcinomas in Ptc1+/− mice and that this natural substance does not cause significant toxicity because the overall survival of the treated mice was unaffected. These data are consistent with the observation in sheep that cyclopamine toxicity is limited to Hh signaling-dependent teratogenicity (2, 29) and with the fact that most normal adult mouse and human tissues appear to have very low expression of Hh target genes (30). Basal cell carcinomas would appear to be good candidates for the cutaneous application of SMO antagonists because our data indicate that direct injection of cyclopamine into mouse basal cell carcinomas induces Fas expression and apoptosis. Thus, it should be possible to design topical formulations of

7 Unpublished observation.
cyclopamine or other SMO antagonists for treating basal cell carcinomas, initially in Ptch1/H11001/H11002 mice and eventually in humans. Our data indicate that cyclopamine inhibits the Hh pathway in basal cell carcinomas, as indicated by down-regulation of the target genes HIP and Gli1 (Fig. 3, A and B). We additionally demonstrate that induction of Fas expression (both the protein and the RNA levels) and consequent activation of the FasL/Fas signaling axis is necessary for cyclopamine-mediated apoptosis because cell death is blocked in vitro.

![Diagram](image)

Fig. 6. Regulation of Fas expression by cyclopamine through the Ras-Erk pathway. A, pathway for cyclopamine-mediated apoptosis. B, In the presence of cyclopamine, the levels of PDGFR-α and phospho-Erk were down-regulated. C, epidermal growth factor (EGF) abrogated cyclopamine-mediated sub-G1 accumulation in ASZ001 cells. D, The protein levels of Fas, FasL, and activated caspase-3 were detected by Western blotting after 36 hours treatment with 2 μM KAAD-cyclopamine in the presence of other reagents or with U0126 alone (10 μM/L). E, Cell viability was assessed by trypan blue analysis. Each experiment was repeated three times with similar results. F, detection of apoptosis by TUNEL assay in ASZ001 cells with Gli1 expression under the cytomegalovirus promoter. Red arrows indicate Gli1-positive cells, and the white arrow indicates TUNEL-positive cells. G, After enrichment of Gli1-overexpressing cells through cell sorting, the protein levels of PDGFR-α, Fas, and caspase-3 were detected by Western blotting.
by anti-FasL antibodies. Thus, it appears that Fas expression is suppressed by the activity of the Ras-Erk pathway in basal cell carcinoma cells, and overexpression of Gli1, PDGFR-α, or active Raf (all downstream of Ptc1) renders ASZ001 cells resistant to cyclopamine-induced apoptosis (Fig. 6, E and F). Furthermore, addition of a mitogen-activated protein kinase inhibitor U0126 alone is sufficient to induce Fas expression and apoptosis in ASZ001 cells (Fig. 6, C and D). We have analyzed promoter sequences of human and mouse Fas genes and found multiple copies of serum response elements and Ras-responsive elements, suggesting that the Ras/Erk pathway can regulate transcription of murine and human Fas directly. These data are consistent with a previous report showing that cyclopamine causes apoptosis in subsets of small-cell lung cancer and medulloblastomas in the presence of low concentrations of newborn bovine serum in which growth factor content is quite low (9, 11). Because Fas is regulated at multiple levels, it will be interesting to determine whether other mechanisms, including altered Fas membrane translocation, could be involved in cyclopamine-mediated Fas up-regulation.

Because cyclopamine exerts its effects through direct association with SMO, tumors with genetic mutations downstream of SMO may not be sensitive to cyclopamine treatment. We have found that cyclopamine does not cause apoptosis in ASZ001 cells with Gli1 overexpression under the cytomegalovirus promoter (Fig. 6, F and G) or in Gli1-transformed RK3E cells. Similarly, cells expressing activated SMO are resistant to cyclopamine (31). Thus, studies on the genetic mutations in specific target tumors could be helpful in predicting the effectiveness of cyclopamine treatment. Effective treatment of tumors with mutations of genes encoding proteins acting downstream of Smo will require identification of novel small molecular weight compounds acting downstream of SMO signaling. However, because most basal cell carcinomas do contain loss-of-function mutations of PTCH1, cyclopamine should represent an effective and specific agent for basal cell carcinoma therapy, as well as for those visceral cancers with Hh signaling activation, which thus far appears to be driven by overexpression of sonic Hh.

In summary, our results indicate that chronic administration of the SMO antagonist cyclopamine is effective in preventing basal cell carcinoma development in vivo. We demonstrate that cyclopamine inhibits Hh signaling and thereby exerts its effects through induction of Fas expression, leading to activation of the FasL/Fas signaling axis and apoptosis. It is likely that SMO antagonists capable of inhibiting Hh activation and inducing Fas expression hold great promise as a mechanism-directed approach for the treatment of basal cell carcinomas.

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