HEDGEHOG SIGNALLING IN COLORECTAL TUMOUR CELLS: INDUCTION OF APOPTOSIS WITH CYCLOPAMINE TREATMENT

David Qualtrough1, Andrea Buda1, William Gaffield2, Ann C. Williams1 and Christos Paraskeva1*

1Cancer Research UK Colorectal Tumour Biology Research Group, Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol, United Kingdom
2Western Regional Research Centre, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA, USA

Hedgehog (Hh) signalling controls many aspects of development. It also regulates cell growth and differentiation in adult tissues and is activated in a number of human malignancies. Hh and Wnt signalling frequently act together in controlling cell growth and tissue morphogenesis. Despite the fact that the majority of colorectal tumours have a constitutively activated canonical Wnt pathway, few previous studies have investigated the expression of Hh signalling components in colorectal tumours. We describe here epithelial cell lineages derived from both nonmalignant colorectal adenomas and colorectal adenocarcinomas that express both Sonic and Indian Hh. Interestingly, these cells also express the Hh receptor Patched and the downstream signalling components Gli1 and Gli3, suggesting autocrine Hh signalling in these cells. To test whether autocrine Hh signalling contributes to cell survival, we treated colorectal tumour cells with cyclopamine, a known inhibitor of Hh signalling. Cyclopamine treatment induced apoptosis in both adenoma- and carcinoma-derived cell lines, which could be partially rescued by further stimulation of Hh signalling. These data suggest that autocrine Hh signalling can increase aberrant cell survival in colorectal tumour cells and may be a novel target for colon cancer therapy using drugs such as cyclopamine.

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The colonic epithelium is a dynamic tissue, undergoing continual renewal throughout adult life via a balance of cell proliferation and cell death. Tumours arising from the colonic mucosa remain a major cause of mortality globally. The majority of colorectal tumours acquire mutations in the Wnt signalling pathway as an early/initiating event in tumorigenesis. These mutations result in constitutive activation of canonical Wnt/β-catenin signalling and the subsequent upregulation of tumour-promoting genes such as c-myc. During embryonic development, the Wnt pathway frequently interacts in tightly regulated networks with other signalling pathways, such as those stimulated by the TGF-β superfamily and the Hh family of signalling proteins, to control growth and patterning.

Expression of Hh proteins has been studied in the developing gut. Ramalho-Santos et al.2 showed, in the mouse, that by 18.5 dpc (birth occurs at around 19.5 dpc) Ihh is expressed throughout the colonic epithelium and Shh expression appears to be confined to crypt epithelial cells. Mice mutant for Ihh exhibit defective stem cell proliferation and differentiation in the intestinal epithelium, and the phenotype in these mice is similar to that observed for mice lacking Tcf-A.3

SHH and IHH expression in the embryonic mouse colon are confined to the epithelial component of the mucosa, whereas expression of Hh target genes, including the Patched receptor, is located in the subepithelial mesenchyme and the smooth muscle layers.4 Other embryologic studies have shown that Hh signalling from the intestinal epithelium is required for the correct specification and patterning of the underlying mesenchyme.5,6

A study in the adult human gut showed expression of SHH primarily in the fundic glands of the stomach.8 Shh mRNA was detected in a small number of epithelial cells at the base of the crypts of the small and large intestine.8

Secreted SHH and IHH signal through binding to the Patched receptor, which, in the absence of ligand, represses the activity of the transmembrane protein Smo. Release of this repression of Smo allows activation of downstream targets through the Gli transcriptional effectors (Gli1–3, mammalian homologues of the Drosophila gene Cubitus interruptus).9 Gli can then act as a transcriptional activator of the pathway target genes.9 The Hh signalling pathway controls epithelial stem cell proliferation in a variety of tissues in both Drosophila and vertebrates in a highly conserved manner.10 Mutations of Patch and Smo (leading to constitutive activation of the pathway) have been implicated as causal factors in a number of human malignancies, including BCC, medulloblastoma, rhabdomyosarcoma, squamous cell oesophageal carcinoma and transitional cell carcinoma of the bladder.11,12 In the developing cerebellum, SHH inhibits terminal differentiation and maintains a high proliferation rate in granule-cell precursors.13 These studies show that deregulation of the Hh signalling pathway can inhibit differentiation and promote tumorigenesis in a number of different tissues.

The effects of deregulated Hh signalling on tumour growth can be reversed by treatment with the steroidal alkaloid cyclopamine. Derived from the lily Veratrum californicum, cyclopamine inhibits cellular responses to SHH signalling.12,13 Sheep grazing on this lily showed a high proportion of birth defects associated with disrupted Hh signalling in their offspring, without apparent harm to the adult animals. Cyclopamine blocks the abnormal cell growth associated with oncogenic mutations of Patch and Smo in fibroblasts,14 inhibits the malignant growth of medulloblastoma cells lacking Patch function15 and inhibits the SHH-dependent growth of small-cell lung cancer cells.16 The lack of adverse effects of cyclopamine exposure in adults thus far described makes it a possible therapeutic agent for tumours associated with deregulated Hh signalling.

Although Shh expression has been reported to be coincident with the stem cell compartment in the adult colonic crypt, previous studies have failed to show activating mutations of Shh, or

Abbreviations: APC, adenomatous polyposis coli; BCC, basal cell carcinoma; dpc, days postcoitum; Hh, Hedgehog; IHH, Indian hedgehog; Ptch, patched; Shh, Sonic hedgehog; Smo, smoothened; Tcf, T-cell factor; TGF, transforming growth factor.

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*Correspondence to: Cancer Research UK Colorectal Tumour Biology Research Group, Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, UK. Fax: +44-117-9287896. E-mail: c.paraskeva@bristol.ac.uk

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inactivating mutations of Ptc in colorectal tumours. In the current study, we show expression of SHH and IHH in both adenoma- and carcinoma-derived cell lines and expression of PTCH, SMO and GLI1 in the same epithelial cells, suggesting the presence of an autocrine signalling mechanism. Using cyclopamine, we show that inhibition of Hh signalling leads to induction of apoptosis in colorectal adenoma and carcinoma cells, which can be partially rescued by further stimulation of the Hh pathway. These findings suggest that the Hh signalling pathway may represent a novel target for therapeutic intervention using cyclopamine.

**MATERIAL AND METHODS**

**Cell lines and culture conditions**

AA/C1 and RG/C2 are adenoma-derived cell lines. AA/C1 was cultured in conditioned medium as described by Williams et al. RG/C2 was cultured in DMEM supplemented with 20% (v/v) FBS (Life Technologies, Paisley, UK). Both cell lines are anchorage-dependent and nonmotogenic in athymic nude mice. JD/FIBS is a colonic fibroblastic cell line derived from the same patient as the AA/C1 epithelial adenoma line and is cultured in DMEM supplemented with 10% (v/v) FBS. The colon carcinoma-derived cell lines CaCo2, HT29 and SW480 were obtained from the ATCC (Rockville, MD) and Cancer Research UK and cultured in DMEM supplemented with 10% (v/v) FBS; all cell lines were cultured as adherent monolayers in 25 cm² tissue culture flasks (Corning Costar, High Wycombe, UK). Care was taken in the routine culture of cell stocks, to avoid cells becoming confluent, which could result in differentiation.

**RT-PCR**

RNA was extracted from 5 × 10⁶ cells using the Qiagen (Chatsworth, CA) RNeasy mini-kit with “on-column” DNase digestion. Single-stranded cDNA was prepared from 10 μg RNA in a 50 μl volume containing 100 pmols oligo-dT, 15 units AMV reverse transcriptase (Promega, Southampton, UK) and 0.4 mM each dNTP, in appropriate buffer. Primers were designed using Lasergene primer design software (DNASTar, Madison, WI) with the exception of positive control primers to the ribosomal protein 36B4, which were described by Lorentz et al. cDNA (5 μl) was amplified in 100 μl PCRs containing 50 pmols each of primer, 200 μM dNTPs and 0.5 units Taq DNA polymerase (Promega) for 35 cycles. To show a lack of genomic carryover, control reactions were carried out on RNA samples that had not been reverse transcribed (representative sample shown for each primer set). The primers used for Ptch span exons 20–22 of the Ptch1 gene. PCR fragments were sequenced to confirm their identity. PCR primer sequences used were as follows: Shh forward 5'-CGACGGG-GACAGCTCGGGAAG-3'; Shh reverse 5'-CTGCCGGGC-CCTCAGTGTGC-3' (product = 477 bp); Ihh forward 5'-GGCG-CGCTTTGACTGGGTGTATTA-3', Ihh reverse 5'-CTTTTGTGAGCGGGGCGTAGG-3' (product = 487 bp); Ptch forward 5'-CGGCGCTGTTCTCAAGGGCTGTCTT-3', Ptch reverse 5'-GTGGGCTGTGCCTGTTTCG-3' (product = 376 bp); Mo forward 5'-ACCCGGGGTCGCTAGTGAAAG-3', Mo reverse 5'-TGGGCCCAAGAGAAGAGAACATC-3' (product = 562 bp); GlI1 forward 5'-TTCGCCCATGCGCCATGGTGAAC-3'; GlI1 reverse 5'-TACATAGCCCCAGCCCATACCT-3' (product = 480 bp).

**Western blot analysis**

Samples of 2 × 10⁶ cells were prepared for Western blotting as described by Williams et al. Antiserum was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) raised against the carboxy terminus of SHH (C-18), IHH (C-15), PTCH (H-267) and SMO (H-300). The antibody for GLI1 was obtained from Abcam (Cambridge, UK). Blots were subsequently probed with anti-α-tubulin (Sigma, Poole, UK) to show equal sample loading.

**RESULTS**

Both colorectal adenoma- and carcinoma-derived cells express not only Shh and Ihh but also the essential Hh signalling pathway components Ptc, Smo and GlI1.

Five human colorectal tumour and one colonic fibroblast (JD/FIBS) cell line were screened for expression of Shh and Ihh using RT-PCR. The identity of all PCR products shown was confirmed by sequencing and BLAST analysis (NCBI, NIH). Two of these lines were derived from nonmalignant adenomatous polyps (AA/C1 and RG/C2) and 3 from adenocarcinomas (CaCo2, HT29 and SW480). mRNAs for both Shh and Ihh were found in all 5 tumour cell lines but not in the colonic fibroblasts or the “no-Rt” controls (Fig. 1a). Western blots using antisera raised against the
carboxy terminals of SHH and IHH show that the 45 kDa precursor proteins of both proteins were expressed in both the adenoma-derived and the carcinoma-derived colorectal epithelial cells (Fig. 1b).

RT-PCR was then used to assay expression of the major downstream components of the Hh signalling pathway, namely, Ptc, Smo and Gli1, in these cell lines. Again, the identity of all PCR products was confirmed by sequencing and BLAST analysis. Expression of the Hh receptor Ptc and the downstream effectors Smo and Gli1 was detected in both adenoma- and carcinoma-derived cell lines as well as in colonic fibroblasts (Fig. 1a). Western blotting was again used to confirm these findings (Fig. 1b) and showed expression of all components in all of the lines assayed. Interestingly, the adenoma-derived cell lines exhibited higher levels of IHH than the carcinoma-derived cell lines but lower levels of PTCH, SMO and GLI1.

Cyclopamine treatment results in reduced cell yield and increased apoptosis in human colorectal tumour cells

Expression of both the Hh ligands and the downstream signaling components Ptc, Smo and Gli1 in colorectal tumour cells suggests autocrine Hh signalling in these epithelial cells. To determine the consequences of blocking this pathway, cells were treated with cyclopamine, a known inhibitor of Hh signalling. Treatments were carried out using 5, 10 and 20 μM cyclopamine (doses previously described14,21) to determine whether this inhibition reduced cell growth and induced apoptotic cell death.

We and others have shown that in colorectal epithelial cell culture the majority of cells that detach from the adherent monolayer and float in the medium have undergone apoptosis (see Material and Methods).22 Because of rapid detachment of cells entering apoptosis, very few apoptotic cells were seen in the attached population. The proportion of cells floating in the medium that are apoptotic remains constant and can be used as a measure of the extent of apoptosis in culture.

Cyclopamine treatment resulted in decreased cell yields and induction of apoptosis in each of the cell lines investigated at all of the tested doses (Fig. 2). Thus, cells derived from both colorectal adenomas and carcinomas are sensitive to the growth-inhibitory effects of this agent.

Following cyclopamine treatment, apoptosis was confirmed in floating cells by studying the proteolytic cleavage of PARP (as shown by Western blotting, Fig. 2b) and identification of apoptotic morphology following acridine orange staining, as described previously24 (data not shown). Figure 2b clearly shows the presence of full-length (116 kDa) PARP in the attached cells but only the 85 kDa cleaved form in the detached, apoptotic population. Following acridine orange staining, a minimum of 90% of floating cells in control cultures were identified as apoptotic and this proportion remained unchanged following cyclopamine treatment. No increase in apoptosis was observed in the attached cell population (≤3%), and no evidence of necrotic cell death was observed, suggesting that the cyclopamine doses used were not toxic. The basal level of apoptosis varies between cell lines, and RG/C2 in particular demonstrates higher spontaneous levels than the other cell lines used, as reported previously.24

We also tested whether adding an excess of ligand to stimulate the Hh signalling pathway could reduce cyclopamine-induced apoptosis. HEK293 cells were transiently transfected with a vector coding for the active amino-terminal SHH protein (SHH-N) as previously described, to produce SHH-N-rich conditioned medium.25 Culturing cells in SHH-N-containing medium did not result in stimulation of cell growth under the conditions used (data not shown). However, the presence of SHH-N resulted in a significant reduction in cyclopamine-induced apoptosis compared to cells cultured in medium from empty vector-transfected HEK293 (Fig. 3a). This finding suggests that cyclopamine-induced apoptosis could be rescued, at least in part, through stimulation of Hh signaling.

To confirm the inhibition of autocrine Hh signalling by cyclopamine in colorectal cancer cells, we used a Gli-dependent luciferase reporter. Figure 3b shows a dose-dependent reduction in Gli reporter activity in response to cyclopamine treatment in SW480. Furthermore, as Ptc is a target of Hh signalling, we would expect it to be downregulated following cyclopamine treatment. Figure 3c shows a clear downregulation of Ptc in the carcinoma cell lines. Regulation of Ptc in the adenoma cell lines AA/C1 and RG/C2 (which have much lower endogenous levels of Ptc) was small but reproducible in 3 separate experiments. Expression of the other components of the Hh signalling pathway was unaffected by cyclopamine treatment (data not shown).
Figure 2.
DISCUSSION

The Hh signalling pathway plays a key role in regulating cell proliferation and survival in a range of tissues and has been implicated in aberrant cell survival in a number of human malignancies. Expression of SHH and IHH in the colon of the mouse embryo is confined to the epithelium, whereas expression of Hh
target genes (including the Ptch receptor) is located in the subepithelial mesenchyme and the smooth muscle layers. Studies have shown that Hh signalling from the epithelium is required for the correct specification and patterning of the underlying mesenchyme. In the adult human colon, expression of Shh mRNA has been demonstrated in a few epithelial cells at the base of normal intestinal crypts, suggesting a role for SHH in the maintenance of the stem cell compartment. Further evidence of a role for Hh signalling in intestinal epithelial cell proliferation comes from targeted disruption of Ihh in the mouse. Ihh is required for normal proliferation of the intestinal epithelium, and the phenotype in Ihh mutants was similar to that observed in Tcf4 mutant mice. Targeted disruption of Tcf4 is neonatally lethal, and the animals show no proliferative compartments in the prospective crypt regions. As a consequence, the neonatal epithelium is composed entirely of differentiated, nondividing cells.

In this study, we demonstrate expression of SHH and Ihh in cell lines derived from both nonmalignant adenomatous polyps and adenocarcinomas of the colorectal epithelium. These cells express not only the ligands but, in contrast to the embryonic mouse colon, also the receptor (Ptch) and downstream components (Smo and Gli1) necessary to transduce the Hh signal. This finding suggests that these tumour cells are capable of autocrine Hh signalling. Although activating mutations of SHH signalling have not been detected in colorectal tumours, our data suggest that the pathway could be erroneously activated by expression of the receptor and signalling components in the epithelium. Interestingly, the lack of Hh ligand expression coupled with the presence of PTCH, SMO and GLI1 in the colon fibroblast cell line JD/FIBS concurs with the in vivo expression patterns observed in the mouse embryo.

The adenoma-derived cell lines exhibited higher levels of Ihh expression than the carcinoma-derived cell lines, which is contrasted by lower expression levels of PTCH, SMO and GLI1. Although these differences are not reflected in the response of the cells to cyclopamine treatment, they may indicate a change in the role of Hh signalling during tumour progression to invasive carcinoma.

Cyclopamine, a known inhibitor of the Hh pathway, was used to test whether Hh signalling influences colorectal tumour cell survival. Treatment of both adenoma- and carcinoma-derived colorectal epithelial cells with cyclopamine reduced cell yield and induced apoptotic cell death.

The CaCo2 cell line showed particular sensitivity to cyclopamaine-induced apoptosis. Although the precise nature of this sensi-
In conclusion, these data provide evidence of expression not only of the Hh ligands but also of the Hh signalling pathway in human colorectal tumour cells. The induction of apoptosis by cyclopamine, a known inhibitor of Hh signalling, suggests a role for autocrine Hh signalling in colorectal tumour cell survival. These data suggest that this pathway may be an important novel target for colon cancer therapy using agents such as cyclopamine.

REFERENCES


In a panel of colorectal carcinoma–derived cell lines in concurrence with our findings. They also found expression of Gli in 4/11 lines but did not detect expression of Ptc1. We have consistently been able to detect Ptc1, Smo and Gli1 in the colorectal cell lines used in this study in 5 separate RT-PCRs and by Western blotting. The reason for this difference is not clear, but expression of Ptc1 may correlate with a subset of tumours that are particularly sensitive (e.g., CaCo2 cells) to treatment with cyclopamine. These observations may be important when selecting patients to determine the therapeutic potential of Hh signalling inhibitors.

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In conclusion, these data provide evidence of expression not only of the Hh ligands but also of the Hh signalling pathway in human colorectal tumour cells. The induction of apoptosis by cyclopamine, a known inhibitor of Hh signalling, suggests a role for autocrine Hh signalling in colorectal tumour cell survival. These data suggest that this pathway may be an important novel target for colon cancer therapy using agents such as cyclopamine.