Biochemical and Biophysical Research Communications 425 (2012) 64–69

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hedgehog pathway regulators influence cervical cancer cell proliferation, survival and migration

Ivana Samarzija, Peter Beard *

Ecole Polytechnique Fédérale Lausanne (EPFL), Department of Life Sciences, Swiss Institute for Experimental Cancer Research (ISREC), 1015 Lausanne, Switzerland

ARTICLE INFO

Article history:
Received 10 July 2012
Available online 20 July 2012

Keywords:
Cervical cancer
Hedgehog signaling
Proliferation
Apoptosis
Papillomavirus

ABSTRACT

Human papillomavirus (HPV) infection is considered to be a primary hit that causes cervical cancer. However, infection with this agent, although needed, is not sufficient for a cancer to develop. Additional cellular changes are required to complement the action of HPV, but the precise nature of these changes is not clear. Here, we studied the function of the Hedgehog (Hh) signaling pathway in cervical cancer. The Hh pathway can have a role in a number of cancers, including those of liver, lung and digestive tract. We found that components of the Hh pathway are expressed in several cervical cancer cell lines, indicating that there could exist an autocrine Hh signaling loop in these cells. Inhibition of Hh signaling reduces proliferation and survival of the cervical cancer cells and induces their apoptosis as seen by the up-regulation of the pro-apoptotic protein cleaved caspase 3. Our results indicate that Hh signaling is not induced directly by HPV-encoded proteins but rather that Hh-activating mutations are selected in cells initially immortalized by HPV. Sonic Hedgehog (Shh) ligand induces proliferation and promotes migration of the cervical cancer cells studied. Together, these results indicate pro-survival and protective roles of an activated Hh signaling pathway in cervical cancer-derived cells, and suggest that inhibition of this pathway may be a therapeutic option in fighting cervical cancer.

1. Introduction

Cervical cancer is the second most common malignancy among women worldwide [1]. Although the incidence of this disease is slowly decreasing in developed countries due to early diagnosis, it remains an important health issue in developing countries where diagnostic programs are still not well established. The primary hit in the etiology of cervical cancer is infection by HPV, a small non-enveloped virus of the family of papillomaviruses. While the presence of HPV is detected in 99% of all cases [2], infection with this agent is not sufficient to cause cancer. Additional cellular changes are needed to bring about complete cellular transformation and carcinogenesis. In order to identify these cellular changes, we investigated the role of the Hedgehog (Hh) signaling pathway in cervical cancer.

The Hh pathway is one of the core developmental signaling pathways whose mutation during development causes congenital malformations [3]. The pathway is activated by binding of the ligand Hh to its receptor patched (Ptch). This binding releases from repression a second receptor, Smoothened (Smo), which moves to the membrane and triggers a series of reactions that result in translocation of transcription activators encoded by glioma associated oncogenes (Gli1, Gli2 and Gli3) into the nucleus and subsequent transcription of target genes.

A role for Hh signaling in certain cancers has been described. There are two different scenarios by which this pathway may be involved in carcinogenesis. One of them is by mutation of pathway components, as exemplified by the mutation of Ptch1 and Smo in basal cell carcinoma, or mutations of Ptch1 and Suppressor of Fused (SuFu) in medulloblastoma and rhabdomyosarcoma. The other scenario is autocrine requirement for Hh ligand, as seen in small-cell lung carcinoma, and cancers of the digestive tract, prostate, breast and liver [4].

It was previously reported that components of the Hh signaling pathway are gradually up-regulated as the normal epithelium progresses to squamous cell carcinoma [5], suggesting a role for Hh pathway molecules in development of this cancer. In the present work, we studied the role of the Hedgehog pathway in cell proliferation, apoptosis and migration, using a panel of cervical cancer cells that either express or not HPV transforming genes, and non-tumorigenic immortalized primary keratinocytes. By employing recombinant Shh ligand and pathway inhibitors, we found that interference with the pathway reduces survival, proliferation and migration of cervical cancer cell lines. We also found that Shh ligand has a promoting role for proliferation and migration of some of the cell-types tested.

* Corresponding author. Address: EPFL SV-DEC, SV-3516, Station 19, CH-1015 Lausanne, Switzerland. Fax: +41 21 693 0720.
E-mail address: peter.beard@epfl.ch (P. Beard).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.07.051

2. Materials and methods

2.1. Cell culture and reagents

The human cervical cancer cell lines C33, SiHa, C4-1, CasKi, and HeLa (American Type Culture Collection, Manassas, VA, USA), as well as the immortalized keratinocyte line HaCaT, were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Amimed, Allschwil, Switzerland) and penicillin and streptomycin. Recombinant Shh protein was purchased from R&D Systems, Minneapolis, MN, USA. Hedgehog pathway inhibitors KAAD-cyclopamine and GANT61 were purchased from Calbiochem, Darmstadt, Germany. Cleaved caspase 3 and Shh (H-160) antibodies were obtained from Cell Signaling, Danvers, MA, USA and Santa Cruz Biotechnology, Santa Cruz, CA, USA, respectively. Reporter Lysis Buffer (RLB) was purchased from Promega, Madison, WI, USA. Protease Inhibitor Cocktail, set III, was obtained from Calbiochem.

2.2. Gene expression analysis by PCR

Cervical cancer cell lines were grown in 6-well plates in complete growth medium. RNA was isolated using the RNeasy Mini-Kit (Qiagen) following the instructions from the manufacturer. cDNA was obtained using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences). Semi-quantitative PCR was done using primers for *SHH* [6], *PTCH1* [7], *SMO* [8], *GLI1* [6], *GLI2* [8], *GLI3* [8] and *GAPDH* [8].

2.3. Detection of Shh protein by Western blot

Cervical cancer and other cell lines were grown in 6-well plates in complete growth medium. Cells were collected in RLB supplemented with protease inhibitors and incubated on ice for 30 min. Isolated protein samples were denatured for 10 min at 95 °C and separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Transfer of the proteins to nitrocellulose membranes was done using iBlot Dry Blotting System (Invitrogen). Western blot analysis was done using the SNAP i.d. protein detection system (Millipore) with antibody against Shh in 0.1% dried milk in TBS-T buffer (0.2 M NaCl, 25 mM Tris, pH 7.5, 0.5 ml/L Tween-20); followed by incubation with anti-rabbit-HRP conjugated secondary antibody. The signal detection was performed with ChemiGlow (Alpha Innotech) and the membranes were then exposed using a Fluor Chem 8900 camera.

2.4. BrdU incorporation assay for cell proliferation

Cells were plated on a 96-well plate at a density of 5000 cells/well and incubated overnight in complete growth medium. The next day, medium was changed to DMEM supplemented with 0.5% FBS. Twenty-four hours later cells were treated with recombinant Shh (100 and 200 ng/ml), or KAAD-cyclopamine (5 and 10 μM), or GANT61 (5, 10, 15 and 20 μM) or an equal volume of DMSO. The treatments were done in DMEM supplemented with 0.5% FBS. BrdU was added to cells 12 h prior to the assay, which was performed 48 h after treatment with the compounds. BrdU detection was done using the Cell Proliferation Biotrak ELISA System (GE Healthcare) as has been described previously [9].

2.5. Immunofluorescence

Cervical cancer cells were plated in 12-well plates on microscope cover glasses (Ø 18 mm, Thermo Scientific) in complete growth medium. Twenty-four hours later cells were treated with GANT61 (20 μM) in DMEM supplemented with 0.5% FBS. Forty-eight hours after treatment cells were washed with PBS and fixed with 4% paraformaldehyde (PFA). Subsequently, cells were washed with PBS, permeabilized and blocked with 0.25% Triton X-100 in 5% powdered milk for 10 min. Primary antibody was added in 1% milk in PBS and incubated for 1 h. Cells were washed three times with PBS-Triton X-100 (PBS-T) for 10 min. Secondary antibody was added in 1% milk in PBS and incubated for 30 min, followed by washing three times for 10 min with PBS-T. Cells were stained with 0.1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole) for 1 min. Finally, they were washed with PBS and distilled water and mounted onto diazabicyclooctane-glycerol (50%). Images were obtained using a Zeiss Axioplan microscope and were acquired with a Zeiss AxioCam MRm camera using Axiovision 4.5 software. The 20× objective was used.

2.6. Wound-closure (scratch) assay

Cells were plated in 12-well plates in complete growth medium. Twenty-four hours later cells were treated with recombinant Shh (500 ng/ml) or GANT61 (20 μM) and a scratch was made through the confluent cell monolayer using 100 μl pipette tip. CasKi cells were imaged 0 and 20 h after scratching, while SiHa cells were imaged 0 and 96 h after scratching. Images were captured with the Olympus System CELLR using a 10× objective.

3. Results

3.1. Cervical cancer cell lines express Hh pathway components

Certain components of the Hh signaling pathway have been reported to be over-expressed in cervical cancer in comparison to normal tissue [5]. To confirm this and to determine its importance, we tested the expression of Hh pathway components using semi-quantitative RT-PCR and a panel of cell-types including cervical cancer-derived cells (C33, SiHa, C4-1, CasKi, and HeLa) as well as the non-tumorigenic immortalized keratinocyte line HaCaT. We found that all components of the pathway tested (Shh, Ptch1, Smo, Gli1, Gli2 and Gli3) were expressed in the cervical cell lines (Fig. 1A). In addition, we confirmed that the Shh ligand is present in all cell lines tested also at the protein level (Fig. 1B). Production of the Hh pathway components appears to be independent of the presence of the HPV genome, because the expression of Hh signaling components was similar in HPV-negative cells (such as C33 cervical cancer cells and the HaCaT immortalized line) to that in cells containing HPV: CasKi, C-41, SiHa and HeLa.

3.2. Inhibition of the Hh pathway reduces proliferation and survival of cervical cancer cells

To test the importance of this pathway for the continued proliferation or survival of cervical cancer cells, we carried out BrdU incorporation assays with and without treatment with pathway activators or inhibitors. A role for the Hh pathway in the growth of a number of cancer cell lines has been suggested. We used Shh as a pathway activator, and two different inhibitors that interfere with the pathway either at the level of the receptor Smo (KAAD-cyclopamine), or the transcription factor Gli (GANT61, Fig. 2). KAAD cyclopamine is a more soluble and more potent cyclopamine derivative, while GANT61 is a small molecule inhibitor that blocks binding of Gli to DNA [10]. We found that addition of Shh ligand increases proliferation of the CasKi cervical cancer line and of HaCaT cells, while inhibition of the pathway at both levels (Smo and Gli) reduces cell numbers and BrdU incorporation for all the cell-types tested (except HeLa where the effect was