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Primary Cilia are Present on Human Blood and Bone Marrow Cells and Mediate Hedgehog Signaling

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Abstract

Primary cilia are non-motile microtubule-based organelles that are present on the cellular membrane of all eukaryotic cells. Functional cilia are required for the response to developmental signaling pathways such as Hedgehog (Hh) and Wnt/ β -catenin. Although the Hh pathway has been shown to be active in leukemia and other blood cancers, there have been no reports describing the presence of primary cilia in human blood or leukemia cells. In the present study, we show that nearly all human blood and bone marrow cells have primary cilia (97–99%). In contrast, primary cilia on acute myeloid leukemia cell lines (KG1, KG1a, K562) were less frequent (10–36% of cells) and were often shorter and dysmorphic, with less well defined basal bodies. Finally, we show that treatment of blood cells with the Hh pathway ligand (SHh) causes translocation of SMO to the primary cilia and activation of Hh target genes, demonstrating that primary cilia in blood cells are functional and participate in Hh signaling. Loss of primary cilia on leukemia cells may have important implications for aberrant pathway activation and response to SMO inhibitors currently in clinical development.

Keywords

Primary Cilia; Hedgehog Signaling; Leukemia

Introduction

Primary cilia are organelles present on almost all the mammalian cells that are critical to the proper function of developmental signaling pathways such as Wnt/ β -catenin and Hedgehog. (1,2) Primary cilia are non-motile and possess a 9+0 arrangement of microtubules, unlike the 9+2 arrangement in the case of motile cilia (3–4). In canonical Hh signaling, pathway ligands (Sonic Hh, Indian Hh, or Desert Hh) bind to the transmembrane protein Ptch1 that is located in primary cilia. This causes PTCH1 to move out of the cilia and allows translocation of SMO to the cilium where it can modulate the stability and processing of the GLI family of transcription factors (GLI1, GLI2, GLI3) that ultimately mediate the

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downstream effects of Hh signaling (5,6). Assembly of primary cilia and movement of proteins within the cilia is dependent on the intraflagellar transport (IFT) system (7). Mice with mutations in key IFT proteins show defects in neural formation and cyclopia, which phenocopies loss of function mutations in the Hh pathway; it was this observation that first suggested a link between primary cilia and Hedgehog signaling (8).

Loss of primary cilia or their function has been linked to a number of human genetic diseases, called ciliopathies (9). Recent studies suggest that abnormal Hh pathway activity due to ciliary dysfunction could lead to various cancers such as glioblastoma, medulloblastoma, basal cell carcinoma, breast, prostate and ovarian cancer (10–12). The Hh pathway has emerged as an exciting new therapeutic target in leukemia and myeloproliferative diseases. Hh target expression has been linked to chemo-resistance and poorer survival in both acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) (13,14). Despite this promise, very little is known about how Hh signaling is activated and regulated during hematopoiesis and malignant transformation. Genetic knock out studies of *Smo* in mouse models of hematopoiesis have shown conflicting results, with some investigators reporting that SMO is critical to normal hematopoiesis, while others have shown no loss of function (15). Moreover, there have been no reports describing the presence of primary cilia in human blood or leukemia cells. In fact, it is largely believed that primary cilia are absent in hematopoietic cells, although this is largely based on conjecture, rather than published evidence. Interestingly, intraflagellar transport (IFT) proteins and Hh signaling have both been implicated in the function of the immune cells and the immunological synapse suggesting a crucial role for cilia in blood cells (16,17). Therefore, to gain a better understanding of how Hh signaling might be regulated in hematopoiesis, or aberrantly activated in leukemia, we sought to identify cilia in blood, bone marrow, and leukemia cells, and determine if they were involved in Hh signaling.

Material and Methods

Human Peripheral Blood Mononuclear Cells (PBMCs) Isolation

Blood was drawn from the healthy volunteer donors in 10ml blood collection tubes. PBMCs were isolated using Ficoll-Paque PLUS, GE Healthcare Life Sciences and density centrifugation per manufacturer's instructions.

Bone marrow

Human bone marrow was obtained from excess material collected from healthy donors donating for allogeneic bone marrow transplant, under the University of Southern California IRB approved protocol. Bone marrow mononuclear cells (BMMCs) were isolated using density centrifugation with Ficoll-Paque PLUS.

Cell Culture

NIH3T3 cells (mouse embryonic fibroblast), leukemic cell lines (KG1, KG1a, K562) were purchased from American Type Culture Collection (ATCC). All the cell lines were maintained were at 37°C under 5% CO₂ levels and 100% humidity. NIH3T3 cells were grown in DMEM media containing 10% fetal bovine serum. All other cell lines were

maintained in RPMI media containing 10% fetal bovine serum. Primary PBMCs and BMMCs were analyzed directly after isolation without culture, except for experiments where PBMCs were treated with recombinant SHh. For these experiment cells were cultured in advanced RPMI with one percent FBS for 24 hours.

Antibodies

Anti-tubulin acetylated (T6793) mouse monoclonal, anti-smoothed (SAB 2500961) goat polyclonal, anti- γ -tubulin (DQ19; T3195) rabbit polyclonal were purchased from Sigma.

Confocal Microscopy

Cells were washed with PBS and were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Fixed cells were washed with PBS, and blocked with blocking buffer (2% BSA, 2% normal goat serum, 2% Donkey serum, 0.1 % Triton X-100 in PBS) for 1hr. All the primary antibodies were diluted in blocking buffer (anti-acetylated tubulin 1:400; anti-gamma tubulin 1:400, SMO 1:200) and used to label cells at room temperature for 1hr. After incubation with primary antibodies, cells were washed with PBS twice. After washing cells were incubated with Alexa Flour conjugated secondary antibodies (anti-mouse-488; anti-rabbit 546, anti-goat 648) for 1 hr. Next, cells were washed with PBS and spread onto gelatin-coated slides. Slides are allowed to air dry and then mounted with vectashield antifade mounting medium containing DAPI. Microscopy was performed on Laser Scanning confocal microscope (Carl Zeiss LSM710). Images were captured at 63X objective with oil immersion.

Image Analysis

All the images were analyzed using Zen 2012 and Imaris software from Bitplane. Intensity plots were created to calculate the individual channel intensity using profile tab of Zen software. Cilia frequency was determined by counting at least 200–300 cells for each type of cells. Primary cilia were counted only if the ciliary axoneme marker acetylated tubulin was presented adjacent to signal from γ -tubulin. Number of ciliated cells was expressed as a percentage of total cells counted.

Results and Discussion

Primary cilia are present on mononuclear cells isolated from human peripheral blood and bone marrow

Primary cilia have been well characterized in the adherent fibroblast cell line, NIH3T3, and we optimized our staining protocols in these cells before attempting to evaluate blood cells for cilia. NIH3T3 cells were grown overnight on coverslips in a six well plate using RPMI media contained 10% FBS. After overnight culture in complete media, cells were serum starved for 48 hr. Cells were fixed with 4% paraformaldehyde and incubated with antibodies against acetylated-tubulin, which identifies the axoneme of the cilia, and gamma-tubulin, which marks centrioles located at the base of all primary cilia. (18) To identify primary cilia in the hematopoietic system, we modified our labeling protocol for non-adherent primary cells. We chose not to serum starve cells because we were interested in knowing if cilia were present in un-manipulated blood and marrow cells, *in vivo*, and did not want to introduce

any artifacts due to culturing of primary cells. After isolation by density centrifugation (Ficoll), we first attempted to Cytospin cells onto glass slides and then proceeded with fixation and labeling as was done for adherent cells. This resulted in distorted morphology and inconsistent identification of cilia. We then modified our protocol to fix with 4% paraformaldehyde and perform primary and secondary antibody labeling in solution. After labeling was complete, cells were placed on gelatin-coated slides and mounted for microscopy. With this modified method we were able to identify cilia on 95–97% of peripheral blood cells and bone marrow cells (Fig. 1A–B, S1–4). Unlike the cilia in NIH3T3 cells, cilia in PBMCs appeared shorter, and many appeared paired. Previous work in cell lines suggest that primary cilia are optimally identified after cells are grown to confluence and serum starved, and cilia length appeared to be proportional to the time of serum starvation (18). However subsequent reports have reported that cilia present in primary tissues are smaller than those described on adherent cell lines (19), suggesting that smaller cilia may be more representative of what is found *in vivo*.

Cilia on blood cells are functional and are involved in Hh signaling

After establishing the presence of primary cilia on blood cells, we wanted to determine if they were functional. Activation of Hh signaling requires movement of SMO into the primary cilia and serves as a good assay for ciliary function. In untreated control NIH3T3 cells, staining for SMO was not concentrated into the cilia. After the treatment with the ligand SHh, which activates the pathway, staining for SMO (magenta) was localized in the primary cilia in NIH3T3 cells (Fig 2A i–ii). We next repeated this treatment with PBMCs and observed localization of SMO in primary cilia after treatment with SHh (Fig. 2A iii–iv). To further confirm the translocation of SMO into the cilia, we analyzed fluorescence intensity of SMO and acetylated tubulin along the axis of the primary cilia. In untreated cells, the SMO signal (magenta) did not conform to the cilia signal (red) demonstrating a lack of co-localization (Fig. 2B). After treatment with SHh, we saw the fluorescence peak for SMO corresponded to that of acetylated tubulin, confirming their co-localization (Fig. 2C). To validate that changes in SMO localization were associated with activation of Hh signaling, we showed that levels of the canonical Hh target gene, *GLI*, were increased after SHh treatment. (Fig 2D)

Loss or disruption of primary cilia is a hallmark of various human cancers associated with abnormal Hh signaling such as basal cell carcinoma, medulloblastoma, breast cancer, and ovarian cancer (10,11). Hedgehog signaling is critical for hematopoietic development and is important for the survival and proliferation for hematological tumors; however there have been no reports describing the presence of primary cilia in leukemia. (16) We examined three myeloid leukemia cell lines for the presence of primary cilia using our staining protocol. In all cases, we were able to identify primary cilia on leukemia cells, however, the frequency was much lower (10–36%) than what was seen in primary hematopoietic cells. (Fig. 3A–C). Since primary cilia have been shown to be required for Hh pathway activation during normal development, the low frequency of cilia seen on leukemia cells suggest that pathway activation in AML may occur aberrantly, and outside the normal signaling paradigm. Further studies to delineate the role of primary cilia in leukemia and their response to SMO antagonists currently in development are needed.

In summary, this is the first report to describe the presence of primary cilia on hematopoietic and leukemia cells. Furthermore, we believe that our solution based primary cilia labeling technique could find use in the study of all suspension cells, and will have broad application in efforts to understand the role of Hedgehog signaling in normal and malignant hematopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BMMCs	Bone Marrow Mononuclear Cells
PBMCs	Peripheral Blood Mononuclear Cells
SMO	Smoothened
SHh	Sonic hedgehog
Hh	Hedgehog

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- Primary cilia are present on most hematopoietic cells
- Primary cilia mediate Hedgehog signaling in hematopoietic cells
- AML cells show reduced frequency of primary cilia with abnormal morphology

Figure 1. Presence of primary cilia in blood mononuclear and bone marrow cells

(A) Confocal Image of primary cilia in NIH3T3 fibroblasts (positive control) using antibodies against acetylated alpha-tubulin (labeling primary cilia, red), gamma tubulin (labeling basal body, green) and DAPI (labeling nuclei, blue) (Top Panel, Magnification 63X, Scale bar 5 μ m). Normal human peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) possess primary cilia (Middle and bottom panel). Mononuclear cells were isolated from peripheral blood or bone marrow and immune-labeled for gamma tubulin (green), acetylated alpha-tubulin (red) and DAPI (blue). Acetylated alpha-tubulin is enriched in cilia axoneme and gamma tubulin is enriched in basal bodies. The spatial arrangement of the staining of these proteins was used to identify primary cilia in PBMCs and BMMCs. (B) Three dimensional (3D) surface projection image of PBMCs created by combining all the Z-stacks. The image shows the presence of primary cilia on the surface of cells (red).

Figure 2. PBMcs are Hedgehog pathway responsive

(A) Hedgehog pathway components display dynamic patterns of ciliary localization in response to sonic hedgehog ligand, SHh. NIH3T3 cells treated with SHh were used as a positive control for SMO localization in primary cilia (Top panel left to right). PBMcs and NIH3T3 cells were cultured in the presence SHh (100ng/ml) for 24h. After completion of the treatment, confocal microscopy analysis was done using antibodies against alpha-acetylated tubulin (labeling primary cilia, red), gamma tubulin (labeling basal bodies, green) and SMO (magenta). SHh mediated Hedgehog pathway activation leads to SMO translocation to primary cilia marked with arrows. (B) Fluorescence intensity profiles of primary cilia in PBMcs. Zen software was used to measure the localization of SMO to cilium in PBMcs (untreated); an arrow was drawn across cilia to evaluate the intensity of different channels. Y-axis represents the fluorescence intensity (arbitrary units) for different channels and X-axis represents the distance (μm). (C) Treatment of PBMcs with SHh (100ng/ml) for 24 h, SMO moves into primary cilia upon Hedgehog pathway activation, arrows were drawn across cilium and the intensity plots was recorded, which shows the presence of SMO in cilium upon SHh treatment. (D) SHh treatment of PBMcs lead to upregulation of *GLI1* transcript levels as measured by quantitative RT-PCR, $*p < 0.05$ compared to control.

Figure 3. Presence of primary cilia on leukemic cells

(A) KG1 cells (top panel), KG1a (Middle panel) and K562 (bottom panel) were serum starved for 48h. After serum starvation cells were stained with alpha-acetylated tubulin (labeling primary cilia, red), gamma tubulin (labeling basal body, green) and DAPI (labeling nuclei, blue). Presence of both the acetylated alpha-tubulin along with gamma tubulin confirms the presence of primary cilia on the KG1, KG1a and K562 cell lines (Magnification 63X). (B) Panel from left to right shows the region of interest (ROI) for KG1, KG1a and K562 cell lines, respectively. (C) Histogram represents the comparison of percentage of ciliated cells between various hematopoietic cells and leukemic cell lines used in the present study. To determine the frequency of primary cilia, the number of cells with cilia were divided with the total number of nuclei counted (200–300) from random selected fields.