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Anti-apoptotic role of sonic hedgehog on blood platelets

Sharda Kumari [a], Susheel N. Chaurasia [a], Kailash Kumar [b], Debabrata Dash [a,*]

[a] Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, 221005, India
[b] Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, 221005, India

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ABSTRACT

Sonic hedgehog (Shh) is an essential morphogen involved in vertebrate organogenesis. Perturbation of Hh signaling is associated with pathological consequences like tumor formation and chronic lung fibrosis. Platelets are highly sensitive circulating blood cells responsible for hemostasis, while hyperactivity of these cells lead to morbidities like ischemic heart diseases and stroke. Despite being terminally differentiated cells with life span of 10-12 days, platelets have recently been shown to respond to Wnt ligand, another developmental signal similar to Shh. In this study, we demonstrate that components of Shh signaling, Patched and Gli3, are expressed in human platelets consistent with existence of functional Hedgehog signaling in these cells. Shh had potent inhibitory effect on platelet apoptosis induced by ABT-737 or thrombin through attenuation of caspase-3 activity. The Shh-mediated pathway may thus represent a novel endogenous mechanism for regulating platelet activity and life span.

Introduction

The Hedgehog (Hh) signaling plays a critical role in vertebrate and invertebrate embryogenesis and determination of cell fate and patterning during organogenesis. Perturbation of Hh signaling is associated with pathological conditions like tumor formation and chronic lung fibrosis [1]. There are three mammalian Hh proteins, with distinct patterns of expression and functions: Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh (Dhh) [2]. Shh is the best studied ligand of the vertebrate pathway. In the absence of Hh ligand, its membrane receptor, Patched (Ptc), maintains smoothened (Smo), a proto-oncoprotein downstream of Ptc, in the inhibited state. Hh pathway is activated when Hh binds to its cognate receptor. This releases the inhibition of Ptc on Smo, allowing transduction of the Hh signal into the cell, and activation of the Hh-responsive transcription factors, glioblastoma-associated protein 1 (Gli1), Gli2 and Gli3. The resulting full length Gli activates their target genes in the nucleus, which is the hallmark of the classical or "canonical" response to Hh [3]. Emerging evidence suggests that Hh has additional functions in the absence of Gli activation, which may or may not be mediated by Smo, and are collectively referred to as "non-canonical". For example, Ptc has a caspase-activating domain which triggers apoptosis in the absence of ligand binding [3].

Shh functions as an essential survival factor in many cell types during development, which include neural stem cells, cells in the neural tube, midbrain, forebrain, neural crest, retina and ventral sclerotome [3]. These findings are supported by the fact that restoring Shh signaling can suppress apoptosis [3]. Shh signaling components expressed in cells of the immune system (B and T lymphocytes and dendritic cells) are known to play key roles in their proliferation as well as survival [1,4–6].

Platelets are anucleate cells with a life span of 10-12 days, which play central roles in hemostasis and pathological thrombus formation associated with ischemic heart diseases and stroke [7]. Recently, the Wnt-β-catenin signaling pathway has been shown to function in human platelets [8,9]. Wnt being a developmental signal, this prompted us to examine whether platelets also react to Hh signaling ligands. Here we have demonstrated that components of the canonical Hh signaling pathway are expressed in anucleate platelets. Strikingly, Shh had significant inhibitory effects on platelet apoptosis induced by ABT-737 as well as by thrombin, demonstrated by inhibition of ROS generation, decreased annexin V binding and stabilized mitochondrial transmembrane potential ($\Delta\psi_m$). The molecular mechanism of Shh action was attributable to significantly attenuated caspase-3 activity in ABT-737-treated platelets. Reports from our lab [10] as well as elsewhere [11] have demonstrated that platelet life span is delimited and regulated by apoptosis. The present study adds to our knowledge on the mechanistic basis of platelet life span regulation and may be relevant to conditions such as thrombocytopenia.

Materials and methods

Materials

FITC-annexin was from Becton Dickinson India Pvt. Ltd. Recombinant Shh was from R & D systems. SuperSignal West Pico chemiluminescent substrate was from Millipore. ABT-737 was from Selleck Chemicals. Primary antibody against Patched and horseradish peroxidase (HRP)-labeled anti-rabbit secondary

* Corresponding author. Tel.: +91 9336910665; fax: +91 542 2367568.
E-mail address: ddash.biochem@gmail.com (D. Dash).

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antibody were from Santa Cruz. Polyvinylidene fluoride (PVDF) membrane was from Millipore India. JC-1, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 2',7'-dichlorfluorescein-diacetate (H_2DCF-DA), human thrombin, apyrase, Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), EGTA, sodium orthovanadate, acetylsalicylic acid and bovine serum albumin (fraction V) were procured from Sigma Aldrich India (P) Ltd. HeLa cell line was obtained from National Centre for Cell Science (*NCCS*), Pune, India. Reagents for electrophoresis were from Merck India. Primary antibody against Gli3 was a gift from Boster Immunoleader. All other reagents were of analytical grade. Milli-Q grade type 1 deionized water (Millipore) was used for preparation of solutions. The study was reviewed and approved by the Institutional Ethics Committee of the University.

Platelet preparation

Platelets were prepared from human blood as previously described [12]. Briefly, peripheral venous blood in citrated–phosphate-dextrose adenine was centrifuged at 180 ×*g* for 20 min. The platelet-rich plasma (PRP) was incubated with acetylsalicylic acid (1 mM) for 15 min at 37° C, followed by ethylenediaminetetraacetate (EDTA) at 5 mM and centrifuged (600 ×*g* for 15 min) to pellet the cells. Platelets were then washed in buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 1 mM ethylene glycol tetraacetic acid (EGTA), 5 mM glucose and 0.6 ADPase units of apyrase/ml, pH 6.2). Cells were finally suspended in buffer B (pH 7.4), which was same as buffer A but lacks EGTA. Cell count was adjusted to 1-4 × 10^9 platelets/ml in the final suspension.

Annexin V binding assay

Platelets (1 x 10^8 cells in 100 μl) were pre-incubated with 3 μg/ml Shh for 30 min at RT and subsequently treated with thrombin (1 U/ml, for 10 min at RT) or ABT-737 (1 μM, for 1 h at 37 °C). Cell suspensions were then centrifuged and resuspended in 1X annexin binding buffer. Platelets were then labeled with 5 μl (equivalent to 15 ng) FITC-Annexin V and incubated for 30 min at RT in the dark and analyzed using flow cytometry. For each sample, 10000 annexin-V positive events were acquired. The extent of annexin-V binding was calculated in stimulated as well as control resting platelet samples from the mean fluorescence intensity using Cell Quest Pro software.

Measurement of intracellular reactive oxygen species (ROS)

H_2DCF-DA detects the generation of ROS by oxidation of dye to fluorescent DCF. Platelets were preincubated with Shh (3 μg/ml) for 30 min at RT and subsequently treated with thrombin (1 U/ml, for 10 min at RT) or ABT-737 (1 μM, for 1 hr at 37 °C) followed by incubation with 20 μM H_2DCF-DA for 20 min at 37 °C. Fluorescent DCF was quantified at 530 nm (excitation, 488 nm) with a fluorescence microplate reader (BioTek model FLx800). Hydrogen peroxide-treated platelets were used as a positive control.

Determination of mitochondrial transmembrane potential

Fluorochrome JC-1 can selectively enter into mitochondria to form aggregates (red). As the mitochondrial transmembrane potential ($\Delta\psi$) collapses, the color changes from red to green due to release of monomeric dye. In order to study $\Delta\psi$, platelets were pre-incubated with Shh (3 μg/ml) for 30 min at RT, and subsequently treated with thrombin (1 U/ml, for 10 min at RT) or ABT-737 (1 μM, for 1 hr at 37 °C) followed by incubation with 10 μM JC-1 for 15 min at 37 ° C in dark. Cells were washed in PBS and JC-1 fluorescence was analysed by flow cytometry analyzed in FL1 and FL2 channels to detect dye monomer and aggregates, respectively. The ratio of red to green (FL2/FL1) fluorescence reflected mitochondrial transmembrane potential. CCCP-treated platelets were used as a positive control.

Western blotting

Proteins were electrophoretically separated on 10 % sodium dodecyl sulphate-polyacrylamide gels and transferred onto a PVDF membrane (0.8 mA/cm^2, 2 h) in a semi-dry blotter (TE 77 PWR, GE Healthcare India). Blots were incubated for 1 h in the presence of 5 % (w/v) BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) to block residual protein binding sites. Subsequently, membranes were incubated overnight at 4 °C with the primary antibody against Patch and Gli3 (1 μg/ml), followed by HRP -conjugated secondary antibody (diluted 1:10,000 in TBST) for 2 h. Specific protein bands were detected by enhanced chemiluminescence using mixture of luminol and peroxide (1:1) and quantity was determined using multi-imaging system (BioSpectrum 800, UVP) and VisionWorks LS Analysis software.

Caspase-3 activity assay

To determine cytosolic caspase-3 activity, ABT-737-treated platelets were pre-incubated with 3 μg/ml Shh and lysed with an equal volume of 2X RIPA buffer (100 mM HEPES, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA and 2 % triton X-100, pH 7.4). After 10 min incubation in ice, equal volume of 2X substrate buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 10 μM caspase substrate AC-DEVD-AMC) was added to each lysate and further incubated for 30 min at 37 °C [13]. Caspase-3 activity was determined from the extent of cleavage of fluorogenic substrate measured at 460 nm emission (excitation, 360 nm).

Statistical methods

One-tailed Student's *t* test was applied for determining significance of differences in the means. *P* values less than 0.05 was considered significant. Data are presented as mean ± SD of at least 3 independent experiments (3 in case of immunoblotting) performed with blood drawn from different donors.

Results

Human platelets express components of Hh signaling pathway, Ptc and Gli3

It was earlier reported that components of hedgehog signaling are expressed in immune cells (B and T lymphocytes and dendritic cells) and endothelial cells [2,4,5]. We examined expression of these proteins in human blood platelets by Western blotting. Fig. 1A and B clearly showed the presence of the Shh receptor Ptc and Gli3 in human platelets, which points to a functional Hh pathway in these cells. HeLa cells are known to express Patched, so we have taken HeLa whole cell lysate as positive control [14,15].

Shh inhibits ABT-737- induced platelet apoptosis

Shh is known to inhibit apoptosis in several cell types including neuroepithelial cells, neurons, retina, lymphoma and dendritic cells [2,3,16–18]. Consequently, we examined the effect of Shh on platelet apoptosis induced by ABT-737. ABT-737 is a BH3-mimetic drug that antagonizes pro-survival Bcl-xL, a key mediator of platelet survival [11]. Pharmacological inhibition of Bcl-xL by ABT-737 leads to apoptosis-like events in blood platelets [10,11] and enhanced clearance of platelets by the reticuloendothelial system [19]. ABT-737 (2.5 μM) induced phosphatidylserine (PS) redistribution from the inner to the outer leaflet of membrane, an early event during apoptosis, the rise in intracellular ROS and collapse of mitochondrial transmembrane