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Cholesterol and its derivatives in Sonic Hedgehog signaling and Cancer

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Abstract

The connection between the Hedgehog pathway and cholesterol has been recognized since the early days that shaped our current understanding of this unique pathway. Cholesterol and related lipids are intricately linked to HH signaling: from the role of cholesterol in HH biosynthesis to the modulation of HH signal reception and transduction by other sterols, passing by the phylogenetic relationships among many components of the HH pathway that resemble or contain lipid-binding domains. Here I review the connections between HH signaling, cholesterol and its derivatives and analyze the potential implications for HH-dependent cancers.

Hedgehog signaling overview

Hedgehog (HH) proteins promote specification, proliferation, differentiation and migration in a variety of cell types. The three HH isoforms, Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH), act on a 12-transmembrane tumor suppressor receptor Patched1 (PTC1) (Figure 1) [1]. A second receptor isoform, PTC2, appears to be less relevant in cancer, increasing tumor susceptibility only in a PTC1 heterozygote background [2]. Binding of PTC1 results in de-repression of the oncogene Smoothened (SMO), a 7-transmembrane G_i protein-coupled receptor [3,4], by means of phosphorylation, ubiquitination and subcellular localization changes [5–7]. Active SMO prevents degradation and promotes activation of the GLI family of transcription factors that mediate HH transcriptional responses [1]. Novel HH functions that are not exerted by GLIs and which are termed “non canonical” have been discovered in the last years and are reviewed elsewhere [8].

Hedgehog in cancer development and maintenance

HH proteins control cell growth, survival and differentiation in a cell type-specific manner and by different mechanisms. HH signaling directly regulates proliferation of cerebellar granule precursors and epidermal basal stem cells [9,10] and indirectly supports other cell types by stimulating angiogenesis [11, 12]. Functional heterozygosity for PTC1 is the cause of Gorlin’s syndrome, characterized by high incidence of the triad basal cell carcinoma (BCC) of the skin, medulloblastoma and rhabdomyosarcoma upon somatic inactivation of

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the wild type PTC1 allele [13, 14]. Sporadic loss-of-function mutations of PTC1 and of Suppressor of Fused (SUFU), an intracellular negative regulator of GLI activity, are strongly associated with medulloblastoma and BCC [15–19] (Figure 2). Similarly, gain-of-function mutations of SMO that render it insensitive to inhibition by PTC1 are usually found in the same types of cancers, in which constitutive, ligand-independent activation of the HH pathway occurs in a cell autonomous manner [20, 21]. Other cancer types, mostly epithelial, are characterized by overexpression of SHH and IHH which act in autocrine (small cell lung cancer, some GI tract cancers) or paracrine ways with the stroma (pancreatic, prostate adenocarcinoma) creating a tumor favorable environment [22–25]. Yet other cancer types are dependent on stroma production of HH ligands for survival, as is the case of B-cell lymphoma [26]. A hallmark of all these cancer types is the activation of GLI target genes, chief among them GLI1 and PTC1, which are used as readouts of HH signaling activity. However, the contribution of non-canonical HH signaling in tumor progression as an anti-apoptotic and pro-angiogenic factor is starting to be appreciated despite being more difficult to study due to the lack of specific readouts [25, 27].

Cholesterol in Hedgehog processing, diffusion, and reception

The role of cholesterol in the HH pathway was early recognized since active HH proteins are covalently modified with cholesterol, making this post-translational modification unique among all known proteins [28]. HHs are synthesized as ~49 KDa precursors which undergo a first signal peptide cleavage upon entering the ER. Then the C-terminal domain of HHs catalyzes an intramolecular splicing reaction that is resolved by addition of cholesterol to a cysteine in the newly created C-terminus. The cholesterol modified N-terminal fragment is further palmitoylated at the N-terminus producing a highly hydrophobic, high activity protein [29]. Cholesterol is absolutely necessary to carry on this cleavage and to yield a protein with a low diffusion rate whose gradients are finely regulated during embryogenesis. Lipid-modified HH proteins remain associated to the plasma membrane upon secretion in the absence of the 12-TM proteins Dispatched A and B, homologous to PTC1, which contain a Sterol Sensing Domain (SSD) [30, 31]. In the current model Dispatched proteins induce HH release from the membrane allowing the formation of different diffusible forms of HH. Water-soluble HHs have been found as part of a multimeric complex with the hydrophilic residues making a shell for the hydrophobic lipid core, but also associated to lipoprotein particles [32, 33]. In humans there is evidence that a small amount of IHH is present in a lipoprotein fraction [34]. Perhaps more relevant, SHH has been detected in exosomes or microparticles that promote angiogenesis [35]. While the role of cholesterol in processing and trafficking of HHs is well established, a recent study suggests that it may not be absolutely required for activity. A collaborative effort among several HH groups demonstrated that HH precursor is fully active in cell culture and, therefore, the role of cholesterol appears to be more important for the establishments of developmental gradients than for homeostasis in adult organisms [36].

Lipid-modified active HH proteins are not soluble in aqueous solutions, yet they signal at a distance from their source. In *Drosophila*, the single HH isoform is found associated with lipoprotein-like macromolecular complexes called lipophorins and reduction of lipophorin levels results in accumulation of HH near its source and impaired signaling at a distance [33]. Remarkably, *Drosophila* PTC not only binds its cognate ligand HH, but also acts as a Lipophorin receptor to promote its endocytosis and to regulate intracellular lipid homeostasis [37]. This study confirmed that lipoproteins have a role in HH distribution and gradient formation but are not required for signal transduction. These findings promoted the search for the presence of HH in human serum lipoproteins. IHH, but not SHH or DHH, was detected in the very low-density lipoprotein (VLDL) fraction of human serum [34]. While this fraction of IHH is not accessible to tissues, VLDL increased survival of endothelial

cells, which are exquisitely dependent on HH ligands for survival [12], suggesting that this circulating pool of HH could have a role in tumor angiogenesis.

Another enigmatic piece of the lipid-HH puzzle is that the LDL receptor family member LRP2, also known as megalin, is specifically required as a SHH co-receptor with PTC1 during early forebrain specification and its deficiency results in holoprosencephaly phenotypes [38, 39]. It is noteworthy to mention the similarities between the HH and Wnt signaling pathways, both utilizing a 7-TM protein of the same GPCR subtype, and both having in some circumstances an LRP co-receptor, which in the case of Frizzleds is LRP-5 or LRP-6 [40].

Since HH proteins are modified with cholesterol, it is not surprising that PTC1 can bind to cholesterol and can promote the efflux of the fluorescent cholesterol derivative BODIPY-cholesterol, speculatively through its sterol-sensing domain, when overexpressed in a heterologous system [41]. However, binding of HHs to PTC1 does not involve the cholesterol moiety; indeed cholesterol-free HH displays high affinity [42]. It seems that binding of HH proteins to PTC1 inhibits its ability to promote cholesterol efflux and elevates intracellular cholesterol [41]. The use of a cholesterol synthesis inhibitor, lovastatin, prevented activation of the canonical HH pathway by SHH, suggesting that accumulation of intracellular cholesterol is necessary but not sufficient for HH signal transduction. Addition of cholesterol alone stimulated SMO accumulation at the plasma membrane, the first step in SMO activation.

Sterols that regulate Smoothed activity

The mechanism of SMO activation when HH proteins bind to PTC1 remains elusive. PTC1 acts catalytically to inhibit SMO through a mediator molecule, which was postulated to be a lipid given the homology of PTC1 with bacterial permeases of the resistance-nodulation-division (RND) family that transport lipophilic toxins [43, 44]. Consistent with this notion it was proposed that PTC1 activity either increases the concentration of an inhibitor of SMO or decreases the level of a SMO agonist. Some oxysterols (OHC), oxygenated derivatives of cholesterol, can bind to SMO cooperatively with two well-known agonists, purmorphamine and SAG, in a site distinct from the binding pocket of the inhibitor cyclopamine [45]. The endogenous side-chain oxysterols 20(*S*)-OHC and 25(*S*)-OHC, but not oxysterols in position 7 (B ring), activate SMO at low micromolar concentrations, and inhibition of their biosynthetic pathway at different steps using simvastatin, zaragozic acid, ketoconazol or triparanol inhibits HH signaling and proliferation in medulloblastoma cells and fibroblasts [45–47]. Moreover, the effect of these compounds on the Hh pathway is highly stereo-selective, with the *S*-configuration of 20-OHC and 25-OHC being the only active epimer for SMO activation [45].

Numerous evidences indicate that PTC1 represses SMO in a cell autonomous fashion. However, Biljsma et al. challenged the dogma by reporting that PTC1 increases the secretion of pro-vitamin D3 and that this compound or a derived metabolite has the capacity to inhibit SMO non-cell autonomously [48]. A summary of the known role of sterol-like compounds on the membrane components of the HH pathway is illustrated in Figure 3.

Targeting Hedgehog in cancer through sterol modulation

The appreciation of the central role of HH signaling in cancer was followed by a high interest in developing safe and specific HH inhibitors of clinical use [49]. Most HH inhibitors target the GPCR SMO and one of them, GDC-0449 or vismodegib, was already given FDA approval for treatment of recurrent, locally advanced or metastatic BCC [50]. However, a report of emergence of resistant disease by SMO mutations in a

medulloblastoma patient treated with GDC-0449 suggests that SMO inhibitors efficacy might be enhanced with inhibitors of other targets in the HH pathway or by combination chemotherapy [51]. Inhibitors of the GLI transcription factors and of HH ligands have been described and are in different phases of clinical trials [49].

Vitamin D efficacy for treatment of HH dependent cancer is also being investigated. Vitamin D or its bioactive form calcitriol significantly decreased proliferation and expression of GLI-target genes in HH-dependent pancreatic cancer, gastric cancer, cholangiocarcinoma and basal cell carcinoma (BCC) cells in vitro [52–55]. Calcitriol also reduced tumorigenesis of Ptc1^{-/-} BCC cell lines in animal models by both activating a VDR-dependent differentiation pathway and by inhibiting SMO in a VDR-independent manner [53].

The use of statins as cholesterol-lowering drugs for cardiovascular disease was followed by anecdotic evidence, confirmed by some controlled studies, that statins appear to reduce the risk of developing colorectal and prostate cancers [56, 57]. Meta-analysis of large-scale randomized trials for primary or secondary prevention of cardiovascular disease with statins did not show the same effect, but the validity of those results was controversial [58]. Statins target 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the synthesis of cholesterol, oxysterols, and 7-hydroxycholesterol (precursor of vitamin D). As it was described before, some mevalonate pathway metabolites are positive regulators of the HH pathway, while vitamin D3 is inhibitory. Thus the overall effect of statins on HH signaling will depend on the nutritional status of individual and will have a maximal inhibitory effect with vitamin D supplementation and low cholesterol intake. It is then plausible that statins can aid in treatment of HH-dependent cancers together with pathway-specific inhibitors if the conditions are maximized to tilt the balance of cholesterol metabolites toward those that negatively regulate the HH pathway.

In summary, the current view of the role of cholesterol derivatives on HH signaling and the promising findings that statins and vitamin D3 may reduce tumor formation and growth of HH-dependent cancers provides a rationale for combining specific HH pathway inhibitors with sterol modulating drugs for the treatment of cancer.

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Highlights

- Cholesterol-modified Hedgehogs act on distant target cells as multimers or bound to lipoproteins
- Activation of HH signaling is involved in development and maintenance of several cancer types.
- Intracellular cholesterol is required in the receiving cell for HH signal transduction
- Two sterol types, oxysterols and vitamin D3 are agonists and inhibitors of SMO, respectively
- Modulation of sterol synthesis could aid specific HH pathway inhibitors in the treatment of cancer

Figure 1.

Overview of the HH signaling network. HH signaling is initiated upon binding of a HH protein to its receptor PTC, which de-represses SMO. In the canonical pathway, SMO activation leads to increased transcriptional activity of the GLI family of transcription factors. GLI-independent actions of PTC and SMO are collectively known as “non-canonical” signaling.

Figure 2. Summary of association of genetic alterations of the HH pathway and cancer type. In the case of SHH and IHH is by overexpression without any reported mutations, for PTC1 and SU(FU) the defect is deficiency or loss-of-function mutations, for SMO is gain-of-function mutations and for GLI is gene amplification.

Figure 3. Role of cholesterol and its derivatives in the HH pathway. Arrows indicate positive interactions, stopped lines indicate inhibition of the component.