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Sonic Hedgehog Is Produced by Follicular Dendritic Cells and Protects Germinal Center B Cells from Apoptosis

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The Hedgehog (Hh) protein family is a group of secreted intercellular signaling molecules that are essential for cell fate and patterning during the development of many organs (1, 2). Hh was first identified in *Drosophila* (3), and several vertebrate homologues have been subsequently discovered (1). In mammals, three proteins comprise the Hh family: Sonic (Shh), Indian (Ihh), and Desert Hedgehog (Dhh); Shh is the best studied (1).

Hh proteins are synthesized as precursor proteins that undergo an internal cleavage catalyzed by the C-terminal portion of the precursor. This process generates a C-terminal fragment that has no other known function and an N-terminal fragment that has all known signaling activity (4, 5). The biologically active N-terminal fragment receives two lipid modifications, cholesterol coupling and palmitoylation. Both modifications facilitate tethering of the protein to the cell membrane of Hh-synthesizing cells, allowing it to function as a short range signaling molecule with neighboring cells (6–8). The N-terminal fragment can be also released from Hh-producing cells by the action of Dispatched (9, 10) and is then free to diffuse and exert its long range activity (11–14).

All Hh proteins share a common signaling pathway, where Ptc is the ligand binder, and Smo transduces the signal into the cell. In the absence of ligand, Ptc exerts an inhibitory effect on Smo activity that is abrogated after Hh binding. In the absence of Ptc, Smo is constitutively active (1, 15). The mechanism underlying Ptc’s inhibitory function is under debate, although recent evidence suggests that permanent physical interaction between Ptc and Smo is not necessary. On the contrary, this inhibition involves the regulation of vesicular trafficking and cytoplasmic transport of small molecules that modulate Smo activity (16–19). Cell surface Ptc expression, in the absence of Smo, may also function to sequester extracellular Hh protein (1–2).

At the end of the Hh signaling pathway are the members of the Gli family of transcription factors, Gli1, Gli2, and Gli3. The specific functions of the Gli proteins are still unknown, although all the activities demonstrated for Gli1 are positive, whereas Gli2 and Gli3 mainly function as positive and negative regulators of transcription, respectively (20–22).

Recent studies have revealed that Hh proteins not only determine patterning and cell fate during embryonic development, but also function in cell fate determination of self-renewing tissues in the adult, such as the hemopoietic and immune systems. Shh has been shown to regulate the proliferation and differentiation of human hemopoietic precursor cells (23, 24). In the thymus, Shh signaling plays an important role regulating thymic cellularity as well as the development of CD4+CD8− thymocytes (25–28). In addition, the effector function of peripheral CD4+ T cells is modulated by Shh (29, 30). However, no evidence of a role for Hh signaling in B cell differentiation and function has been described. In this study we analyze the expression of Shh in peripheral lymphoid organs and show that Shh is produced by follicular dendritic cells (FDCs), mainly in germinal centers (GCs). GCs are specialized microenvironments comprised primarily of B cells, FDCs, tingible body macrophages, and some T cells. In the GCs, B cells efficiently undergo clonal expansion, somatic hypermutation, and selection to differentiate into memory B cells capable of producing high affinity Abs or into plasma cells secreting Ig (31–34). We also report that GC B cells express Hh receptors and that their survival and function are altered after inhibition of the Hh signaling pathway. In addition, Shh protects GC B cells from Fas-induced apoptosis. Our results indicate, therefore, that Shh...
signaling is involved in the FDC-mediated rescue of GC B cells from apoptosis.

Materials and Methods

Mice and immunizations

Young adult female BALB/c mice were purchased from Harlan Iberica and maintained under pathogen-free conditions. Mice were immunized s.c. with 100 μg of OVA precipitated in alum or i.p. with 10^5 SRBC.

Histology and immunofluorescence

Lymph nodes (LN) and spleens were obtained before and 7–10 days after immunization, embedded in OCT compound (Miles), and frozen in liquid nitrogen. Cryosections (7 μm thick) were air-dried for 2 h at room temperature and fixed in acetone for 10 min. Non-specific binding of Abs and streptavidin was blocked by incubation with diluted donkey serum (Santa Cruz Biotechnology) and avidin-biotin (Vector Laboratories). Shh protein was detected using specific goat Abs against either the C-terminal fragment of Shh (R&D Systems) or the N-terminal domain of Shh (Santa Cruz Biotechnology), obtaining similar results. Next, tissue sections were sequentially incubated with FITC-conjugated multiadsorbed F(ab')2 of donkey anti-goat IgG (Jackson ImmunoResearch Laboratories) and anti-FDCs (FDC-M2; AMS Biotechnology) or anti-Shh (BD Biosciences) mAbs, followed by Texas Red-conjugated multiadsorbed F(ab')2 of donkey anti-rat IgG (Jackson ImmunoResearch Laboratories). In some cases, to detect GCs the sections were also incubated with biotin-conjugated peanut agglutinin (PNA; Sigma España) and streptavidin-conjugated 7-amino-4-methylcoumarin-3-acetic acid (Vector Laboratories). Slides were mounted in Vectashield (Vector Laboratories) and examined on an Axioplan-2 microscope (Zeiss) from the Centro de Microscopia y Citometria, Complutense University of Madrid.

PCR analysis

RT-PCR was performed as previously described (26). RNA isolation was performed using a Stratagene Total RNA Miniprep kit (Stratagene), including a DNAse I digestion step, as recommended by the supplier, to avoid genomic contamination. Total cDNA was synthesized with Superscript II RT polymerase (Invitrogen Life Technologies). Primers were purchased from Sigma-Genosys. Amplifications were performed using the following primer sets and annealing conditions: Ptc, forward primer, 5'-TGGCTCCTGCACTTTCTTAGTG-3', reverse primer, 5'-GCCAGAGCCGCAAGAGATAATTAC-3'; SMO, forward primer, 5'-GCCAGAGGCTGGTAC-3'; reverse primer, 5'-GCCAGAAAGGTGGAGACTA-3' (1°C; 322 bp). Primers were designed from sequences available from the GenBank database (accession no: Ptc, XM_008959; SMO, XM_008959). Primers to detect G3PDH and Shh were previously described (29, 35). All PCRs were performed on a MasterCycler gradient machine (Eppendorf) using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 3 min at 94°C, 40 cycles of 45 s at 94°C, 45 s at each particular T annealing 45 s at 72°C, followed by 10 min at 72°C. PCR products were resolved on a 2% agarose gel, and the expected sizes were measured.

Isolation of cell populations

GC cells were isolated from the spleens or LNs of mice 7–10 days after primary immunization with SRBC or OVA, according to the procedure described by Kosco et al. (36). Briefly, after enzymatic digestion with collagenase and DNase I (Sigma España), cells were isolated on Percoll (Pharmacia Biotech) gradients, and adherent cells were removed after a 1-h adherence step at 37°C. As previously reported, the resulting population consisted of 75–85% B cells, 5–10% T cells, 5–10% FDCs, and <5% tingible body macrophages (36–38).

For isolation of GC B cells, GC cell suspensions were stained with anti-B220-PE mAb (BD Biosciences) and purified by magnetic sorting using AutoMACS (Miltenyi Biotec) in conjunction with anti-PE Microbeads (Miltenyi Biotec) according to the manufacturer’s instructions.

Flow cytometry

To analyze the expression of Ptc and Smo receptors on GC B cells, three- and four-color immunofluorescence stainings were performed by incubating GC cells in PBS containing 1% FCS and 0.1% NaN3, in the presence of saturating amounts of anti-Smo-PE and anti-B220-alkaline phosphocyanin Abs and FITC- or biotin-conjugated PNA, followed by streptavidin-Texas Red for 30 min at 4°C. In some experiments, cells were then treated with a FACS permeabilization solution according to manufacturer’s instructions (BD Biosciences), stained with anti-Ptc-FITC Ab, recognizing an intracellular domain at the N terminus of Ptc, for 30 min, and fixed (BD Biosciences). In all experiments, unconjugated anti-CD16/32 mAb was also added to the Ab mixture to minimize FcR-dependent background staining. Cells were analyzed in a FACSCalibur (BD Biosciences) from the Centro de Microscopia y Citometria, Complutense University of Madrid.

Results

**Shh expression on FDCs and up-regulation in GCs**

The expression of Shh was studied on sections of murine secondary lymphoid organs. Shh was highly expressed in GCs of LNs and spleen from immunized mice. Interestingly, Shh expression was associated with the reticulum formed by FDC-M2-positive FDCs appearing in the light zone (Fig. 1, A–D). The double labeling of tissue sections with anti-Shh and anti-B220 Abs did not show the existence of overlapping areas, indicating that B lymphocytes do

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not express Shh in GCs (Fig. 1E). To further investigate the expression of Shh in the FDC network found in primary follicles, an identical histological analysis was performed in LNs and spleen obtained from unimmunized mice housed in specific pathogen free-conditions. In contrast to the FDC network of GCs, only a few FDCs expressed Shh in the primary follicles (Fig. 1, F and G). Similarly, in CD28-deficient mice, which are unable to form GCs (42), a low number of FDCs weakly expressed Shh (data not shown).

The expression of RNAs encoding Shh was assessed by RT-PCR. We found Shh RNA transcripts in FDC-enriched cell populations from secondary lymphoid organs (Fig. 2A). In contrast, we were unable to detect Shh encoding RNAs in GC B cells (Fig. 2A).

**Hh receptors are expressed by GC B cells**

Because FDCs expressed high levels of Shh in GCs, the expression of Ptc and Smo, both components of the Hh receptor, was studied in B cells maturing in GCs. RNAs specific for Ptc and Smo were detected in GC B cells (Fig. 2A). Flow cytometry revealed that all B220⁺PNAhigh GC B cells expressed Ptc, the component of Hh receptor that binds Shh, whereas ~30% coexpressed Smo, the signaling component of Hh receptor (Fig. 2B). Thus, all GC B cells are capable of binding Hh proteins at their cell surface, and ~30% are capable of transducing the Hh signal into the cell.

Modulation of the expression of Hh receptors on GC B cells in response to different activating stimuli was also analyzed. After 4 h, anti-CD40 and anti-IgM stimulations significantly increased the proportions of Smo⁺ GC B cells. The percentage of Smo-expressing B220⁺PNAhigh cells increased ~40–50% after CD40 ligation and IgM cross-linking, whereas stimulation with LPS hardly affected the proportion of Smo⁺ GC B cells (Fig. 2C). The percentage of Ptc-positive GC B cells did not change significantly after stimulation (data not shown).

**Inhibition of Hh signaling affects proliferation and Ab production by GC B cells**

We investigated the effects of Shh deprivation on the proliferation and Ab production by GC B cells. For this purpose, GC cells were isolated and cultured for 1, 4, and 7 days in the presence of the steroidal alkaloid cyclopamine or the neutralizing anti-Shh mAb 5E1, both inhibitors of the Hh signaling pathway (40, 43, 44). As previously described (36), these cultures could maintain B cell proliferation for several days, although after 7 days the proliferation was reduced by 50%. Blockade of Hh signaling by the addition of cyclopamine or anti-Shh mAb caused a decrease in the proliferation of GC B cells during the first days of culture, but only slight differences could be detected by day 7 of culture (Fig. 3A).

When GC cells were cultured in the presence or the absence of cyclopamine for 7 days, the supernatants were collected, and the Ig isotypes. The addition of cyclopamine significantly reduced IgM, IgG1, and IgG2b titers by 20–30%, whereas IgG2a and IgG3 secretion was not affected by cyclopamine (Fig. 3B).
Hh signaling blockade affects GC B cell viability

Given that Shh has been described as an antiapoptotic factor for several cell types (29, 45–49), we decided to examine whether Shh also affected the survival of GC B cells. Isolated GC cells were cultured in the presence or the absence of Hh signaling inhibitors, and the proportion of annexin V-positive dying cells was assessed in the B220<sup>+</sup>/PNA<sup>high</sup> GC B cell population. After 12 h, inhibition of Hh signaling significantly increased the proportion of apoptotic GC B cells. As shown in Fig. 4, the addition of anti-Shh mAb and cyclopamine increased the percentages of annexin V-positive GC B cells by ~45 and 75%, respectively. These results indicate that endogenous Shh produced by FDCs is a survival stimulus for GC B cells.

Shh prevents Fas-induced apoptosis of GC B cells

GC B cells rapidly undergo apoptosis in vitro, and this process is enhanced after Fas ligation (33, 50–52). To investigate whether exogenous Shh has the ability to maintain the survival of GC B cells, we cultured isolated GC B cells in the presence of anti-CD95 mAb and different concentrations of Shh. After 12 h, ~30% of B220<sup>+</sup>/PNA<sup>high</sup> cells were annexin V-positive dying cells. The proportion of apoptotic cells increased after ligation of Fas, but this effect was counteracted by Shh. The apoptosis-inhibiting effect of Shh was concentration dependent and maximal at 100 ng/ml (Fig. 5). In addition, the survival effect of the highest dose of Shh was similar to that from other apoptosis-inhibiting stimuli, such as anti-CD40 and anti-IgM cross-linkings (Fig. 5). Hence, Hh signaling plays a role in rescue from CD95-induced apoptosis.

Discussion

In this study we report that the Hh signaling pathway plays a role in the Ag-driven B cell differentiation that occurs in GCs. Shh is strongly expressed by the FDC network found in GCs, whereas in the primary follicles the expression of Shh is much weaker and is restricted to a few FDCs. These data suggest that up-regulation of Shh production by FDCs is dependent on antigenic stimulus and interaction with Ag-activated B cells. During the GC reaction, Ag-activated B cells move into the FDC network and replace the primary resting B cells that are initially located within the FDC reticulum of the primary follicles (32, 53). Up-regulation of the expression of several surface cell markers, i.e., FDC-M1, FcγRII/CD23, FcγRII/CD32, VCAM1/CD106, MAdCAM-1 (54–57), and some soluble factors such as IL-6 and FDC-SP (38, 58), is a common feature of the development of the FDC network after immunization and reflects the involvement of those molecules in the maturational events associated with GCs. These membrane Ags and secreted proteins constitute indicators of FDC maturation and activation, and Shh could now be considered a new member of this group.

The fact that GC B cells express the two components of the Hh receptor, Ptc and Smo, indicates that GC B cells are cell targets for
Shh produced by FDCs. Furthermore, the expression of Smo, the Hh signal transducing receptor, is rapidly up-regulated upon GC B cell activation with anti-IgM and anti-CD40 mAbs (which mimic the physiological signals that B cells receive in vivo). This up-regulation of Shh expression after GC B cell stimulation suggests the existence of a direct link among the BCR, CD40, and Hh signaling pathways.

We show that the main role of FDC-derived Shh is to act as a survival factor for GC B cells, because the proportion of apoptotic GC B cells increases after Hh signaling inhibition, and exogenous Shh addition rescues GC B cells from Fas-induced apoptosis. Our results also show that proliferation and Ig secretion by GC B cells are reduced when Hh signaling is inhibited, but these effects are probably the consequence of the decreased GC B cell survival observed. Antiapoptotic effects of Shh have been also demonstrated during development of the nervous system (45), teeth (59), and external genitalia (46), as well as in somite cells (48), keratinocytes (49), thymocytes (60), T lymphocytes (29), and certain neuron populations (47). In GCs, three types of stimuli seem to prevent apoptosis in GC B cells: ligation of the BCR, ligation of CD40, and unknown signals delivered by FDCs (33). All these stimuli have been described to sustain the expression of the long isoform of cellular FLIP<sub>L</sub> (cFLIP<sub>L</sub>), which inhibits the Fas-induced activation of caspase 8 (33, 50, 52). When GC B cells are isolated and removed from their microenvironment, cFLIP<sub>L</sub> is quickly degraded, resulting in the activation of caspase 8 and, subsequently, caspase 3. In contrast, interaction with FDCs maintains high levels of expression of cFLIP<sub>L</sub>, preventing activation of caspase 8 and protecting these cells against Fas-induced cell death (33, 50, 52). The FDC-derived, antiapoptotic signal acts independently of CD40-mediated signals, BCR, or adhesion molecules, such as ICAM-1 or VCAM (51). It is tempting, therefore, to speculate that Shh could be the FDC-produced factor involved in the maintenance of cFLIP<sub>L</sub> expression and the prevention of Fas-induced apoptosis in high affinity GC B cells while they are trapped in the FDC network.

Taken together, the current data provide evidence that the Hh signaling pathway is involved in GC reactions. Shh seems to be a FDC-derived antiapoptotic signal contributing to the prevention of B cell death in the GC in conjunction with BCR- and CD40-derived survival signals. Additionally, upon CD40 and BCR ligation, cell surface Smo expression on GC B cells is up-regulated, thereby increasing the ability of these cells to respond to Shh.

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**References**


