A dual role for Sonic hedgehog in regulating adhesion and differentiation of neuroepithelial cells

Artem Jarov, a Kevin P. Williams, b Leona E. Ling, b Victor E. Koteliansky, b Jean-Loup Duband, a, * and Claire Fournier-Thibault a

a Laboratoire de Biologie du Développement, CNRS et Université Pierre et Marie Curie, 9 quai St.-Bernard, 75252 Paris Cedex 05, France
b Biogen Inc., 14 Cambridge Center, Cambridge, MA 02142, USA

Received for publication 10 April 2003, revised 23 May 2003, accepted 27 May 2003

Abstract

In vertebrates, the nervous system arises from a flat sheet of epithelial cells, the neural plate, that gradually transforms into a hollow neural tube. This process, called neurulation, involves sequential changes in cellular interactions that are precisely coordinated both spatially and temporally by the combined actions of morphogens. To gain further insight into the molecular events regulating cell adhesion during neurulation, we investigated whether the adhesive and migratory capacities of neuroepithelial cells might be modulated by Sonic hedgehog (Shh), a signaling molecule involved in the control of cell differentiation in the ventral neural tube. When deposited onto extracellular matrix components in vitro, neural plates explanted from avian embryos at early neurulation readily dispersed into monolayers of spread cells, thereby revealing their intrinsic ability to migrate. In the presence of Shh added in solution to the culture medium, the explants still exhibited the same propensity to disperse. In contrast, when Shh was immobilized to the substrate or produced by neuroepithelial cells themselves after transfection, neural plate explants failed to disperse and instead formed compact structures. Changes in the adhesive capacities of neuroepithelial cells caused by Shh could be accounted for inactivation of surface integrins combined with an increase in N-cadherin-mediated cell adhesion. Furthermore, immobilized Shh promoted differentiation of neuroepithelial cells into motor neurons and floor plate cells with the same potency as soluble Shh. However, the effect of Shh on the neuroepithelial cell adhesion was discernible and apparently independent from its differentiation effect and was not mediated by the signaling cascade elicited by the Patched-Smoothened receptor and involving the Gli transcription factors. Thus, our experiments indicate that Shh is able to control sequentially adhesion and differentiation of neuroepithelial cells through different mechanisms, leading to a coordinated regulation of the various cell interactions essential for neural tube morphogenesis.

© 2003 Elsevier Inc. All rights reserved.

Keywords: Avian embryo; Neuroepithelium; Morphogenesis; Cell adhesion; Cell migration; Integrin; Cadherin; Sonic hedgehog

Introduction

During development of the vertebrate embryo, neurulation proceeds as a series of steps by which the flat neural plate is shaped, elongated, and bent to form a tube that extends through the entire length of the antero-posterior axis (Colas and Schoenwolf, 2001; Smith and Schoenwolf, 1997). This process allows the positioning and differentiation of the progenitors of the different neuronal and glial cells that ultimately compose the spinal cord. The extensive remodeling of the neural plate necessitates sequential changes in cellular interactions that are precisely coordinated both spatially and temporally. Thus, changes in cell adhesion between neighboring neuroepithelial cells and with the underlying basement membrane occur throughout neurulation, during bending of the neural plate, fusion of the neural folds, and late shaping of the neural tube. During neural tube closure, N-cadherin, a member of the cadherin family of cell-adhesion molecules, is increased in neuroepithelial cells concomitant with the gradual decrease of E-cadherin and occludin, a component of tight junctions,
suggesting an overall disappearance of the epithelial nature of these cells (Aaku-Saraste et al., 1996; Duband et al., 1988; Hatta et al., 1987; Thiery et al., 1984). Later, at the time of neural differentiation, while cells in the floor plate retain their epithelial structure, neuron progenitors lose contact with the luminal side of the neuroepithelium after commitment and become located at the basal side. Neural crest cells represent the ultimate degree of cell movement within the neural tube as, unlike all other neuroepithelial cells, they delaminate from its dorsal side and disperse through the adjacent tissues.

During the last decade, a large number of in vivo and in vitro studies in various animal systems have identified the secreted signaling molecules of the hedgehog and bone morphogenetic protein (BMP) families as key regulatory elements of cellular interactions during neural tube morphogenesis. Opposite gradients of antagonistic activities of BMPs and Sonic hedgehog (Shh) have been shown to direct specification and subsequent differentiation of the neuronal and glial cell types along the dorsoventral axis of the neural tube (reviewed in Ingham and McMahon, 2001; Jessell, 2000; Lee and Jessell, 1999). Shh and BMPs also participate to the control of cell numbers during neural tube morphogenesis, by acting sequentially and locally as mitogenic, trophic, or pro-apoptotic factors (Charrier et al., 2001; Golden et al., 1999; Graham et al., 1994; Rowitch et al., 1999).

The question remains unclear about the modulation of cell adhesion during neurulation. Disruption of cell adhesion molecules during neural tube development, e.g., using antibodies to N-cadherin or antisense oligonucleotides to integrins, emphasize their importance during this process (Bronner-Fraser et al., 1992; Kil et al., 1996). However, limited information is available concerning the regulation of cell adhesion molecules by extrinsic factors during the development of the spinal cord. Delamination of neural crest cells from the dorsal neural tube is triggered by a balance between the activities of BMP-4 and its antagonist noggin, which modifies the expression and activity of integrins and cadherins responsible for intercellular detachment, cytoskeletal changes, and cell motility (Delannet and Duband, 1992; Sela-Donenfeld and Kalcheim, 1999). Furthermore, when challenged with BMPs, ventral neural tubes can generate cells that display characteristics of migrating crest cells (Basler et al., 1993; Liem et al., 1995), indicating that all neuroepithelial cells retain at least transiently the potential to migrate and that cells situated in the ventral neural tube are prevented to do so presumably due to the activity of inhibitors.

We have postulated that, by analogy with their antagonistic activities for driving the differentiation of dorsal and ventral neural tube cells, BMPs and Shh would also exert opposite effects on the adhesive properties of neural epithelial cells during neurulation. While BMPs would favor dispersal of dorsal cells, Shh would instead restrict migration and maintain strong cell-cell cohesion among ventral neuroepithelial cells. To this aim, we have investigated in a preliminary study the effect of ectopically applied Shh on neural crest cell dispersion in vitro, and we found that, in agreement with our hypothesis, Shh blocked crest cell adhesion and migration, essentially by affecting integrin function (Testaz et al., 2001). In the present study, we further extend these observations and we show that the effect of Shh on cell adhesion is not restricted to neural crest cells but also applies to ventral neuroepithelial cells. Moreover, we provide evidence that Shh is able to control sequentially adhesion and differentiation of neuroepithelial cells through different mechanisms, leading to a coordinated regulation of the various cell interactions essential for neural tube morphogenesis.

Materials and methods

Soluble proteins, antibodies, and metabolic agents

Production and purification of soluble human Shh, the Shh(polyQ) variant with the N-terminal residues 32–38 (KRRHPKK) mutated to QQHPQQ, and the truncated Shh (N-9/C-3) variant were described elsewhere (Williams et al., 1999). The mouse monoclonal antibody (mAb) ASC1 raised against a Shh-Fc fusion protein was described previously (Testaz et al., 2001; Williams et al., 1999). The mAbs 5E1 to Shh, 39.4D5 to Islet 1/2, 4C7 to HNF3β, and BEN were all obtained from the Developmental Studies Hybridoma Bank (University of Iowa). The mAbs to N-cadherin, either nonblocking (ID-7.2.3) or blocking (CC-II), were purchased from Sigma. Rabbit polyclonal antibodies to the β1-integrin subunit were provided by K. Yamada. Forskolin was from Sigma and cyclopamine was a gift from W. Gaffield.

Culture of neural plate tissue

Japanese quail embryos were incubated at 38°C until stage 9 of Hamburger and Hamilton (HH; Hamburger and Hamilton, 1951), collected into phosphate-buffered saline (PBS), and the caudal region at the level of the opened neural plate was excised and transferred into a 2.4 U/ml dispase II solution (Roche) for 20 min at ambient temperature. The neural plate was then isolated freed from surrounding tissues, and the neural folds were discarded. The remaining ventral-intermediate portion of the neural plate was explanted onto culture dish coated with 5 μg/ml bovine plasma fibronectin (FN; Sigma) or 20 μg/ml mouse laminin-1 (LN1; Sigma), and cultured in Dulbecco’s minimum Eagle medium (DMEM) supplemented with 3% FN-depleted calf serum (Life Technologies). Cultures were observed with a Nikon Diaphot inverted microscope equipped with a camera connected to a computer and data were analyzed by using Lucia (Nikon) or Metamorph (Universal Imaging Corporation) softwares. The relative increase of
the surface of the explants over time in culture was measured taking for reference the initial surface. Video-microscopy analyses were performed in Terasaki plates essentially as described previously (Dufour et al., 1988).

**Assays for cellular adhesion**

Assays for cellular adhesion of neural plate cells were performed essentially as described previously for neural crest cells (Delannet and Duband, 1992). Cells from freshly isolated neural plate explants obtained from HH stage 9 embryos were dissociated into a single cell suspension using a trypsin-EDTA solution (Life Technologies), pelleted and resuspended in DMEM supplemented with 3% serum, except for adhesion assays in the presence of divalent cations in which PBS was substituted for DMEM. Aliquots of cell suspension containing approximately 2000 cells were deposited in culture dishes coated with FN at 2 μg/ml or LN1 at 20 μg/ml. For adhesion assays in the presence of metabolic agents, cells were preincubated for 15 min with forskolin or cyclophilamine at the final desired concentration before plating. Cells were then incubated at 37°C for 1 h and fixed. Spread cells were counted and results were expressed as the percentage of spread cells in relation to the total number of cells initially deposited on the substrate.

**In ovo electroporation**

The following plasmid constructs under the control of the cytomegalovirus promoter were used for electroporation: pRKS5 expression vector containing the full-length mouse Shh cDNA (provided by C.-M. Fan), alkaline phosphatase (AP) cDNA cloned in pcDNA3 (provided by T. Jaffredo), and EGFP-N1 (Clontech). A plasmid solution containing a mix of Shh or AP cDNA at 1 μg/μl with Green Fluorescent Protein (GFP) cDNA at 0.25 μg/μl was deposited with a micropipette into the lumen of the neural plate of chick embryos at HH stage 9. Electrodes were applied on the vitelline membrane on either side of the truncal region at the level of the segmental plate and five square pulses (30 V, 50 ms length) were delivered bilaterally with reversed polarity in order to electroporate both sides of the neural plate. Eggs were sealed and reincubated for an additional 12 h. Embryos were then dissected out and the electroporated immature neural epithelium, which is just at the onset of the neural plate is still wide open and can be considered as an anteriorly to the Hensen’s node, and explanted into dishes coated with FN or LN1 substrates. At this axial level, the neural plate is still wide open and can be considered as an immature neural epithelium, which is just at the onset of the neurulation process and has not yet been subjected to extensive Shh signaling (Roelink et al., 1994; Yamada et al., 1993).

**Results**

**Adhesion and dispersal capacities of neural plate cells in vitro**

Cell-cell and cell-substrate adhesiveness within tissues cannot be quantitated conveniently in situ and have to be evaluated under in vitro conditions. Thus, to approach the possible role of Shh on the adhesion and migration capacities of neuroepithelial cells, we have developed an assay of truncal neural plate explants cultured for up to 72 h on extracellular matrix molecules. Neural plates were collected from the caudal part of quail embryos at HH stage 9, slightly anteriorly to the Hensen’s node, and explanted into dishes coated with FN or LN1 substrates. At this axial level, the neural plate is still wide open and can be considered as an immature neural epithelium, which is just at the onset of the neurulation process and has not yet been subjected to extensive Shh signaling (Roelink et al., 1994; Yamada et al., 1993).

When cultured on FN substrates, neural plate explants rapidly adhered to the bottom of the dish and, within less than 12 h, numerous neuroepithelial cells have spread onto the substrate at the periphery of the explant (Fig. 1A). During the first day in culture, the explant gradually flat-
tended onto the dish (Fig. 1B), resulting in a 20-fold increase of its apparent surface after 36 h (Fig. 2C). Such a rapid expansion could not be simply attributed to cell proliferation, as the total cell number increased about 3-fold during the same time period. During the second day in culture, expansion of the explant stabilized (Figs. 1C and 2C) and, 3 days after explantation, the neural plate had generally flattened entirely on the substrate and organized as a dense

Fig. 1. Effect of Sonic hedgehog (Shh) on cell adhesion and migration in neural plate explants. Comprehensive views of neural plates cultured for 12 h (A, E, and I), 24 h (B, F, and J), 48 h (C, G, and K), and 72 h (D, H, and L) on fibronectin (FN) substrates in the absence of exogenous Shh (control; A–D), on FN in the presence of Shh added at 2 μg/ml to the culture medium (soluble Shh; E–H), or on FN in the presence of Shh adsorbed onto the substrate at the coating concentration of 20 μg/ml (adsorbed Shh; I–L). Arrows point at neurites sprouting out of the explants. Bar = 100 μm.
pseudo-epithelial sheet surrounded by a few scattered cells (Fig. 1D). Neural plates exhibited essentially the same dispersal behavior when plated onto LN1 substrates (data not shown). Video-microscopy analyses of the explants during the first day in culture (Fig. 2A) revealed that expansion was minimal during the first 6 h, corresponding to the time necessary for the explant to adhere to the dish. From this time on, the rate of expansion of the explant area became maximal and was constant during the subsequent 24 h (Fig. 2A and C). During this process, individual cells crawled on the bottom of the dish while remaining attached to their neighbors and the whole population expanded uniformly as a sheet. These observations therefore indicate that neuroepithelial cells from immature neural plates exhibit the ability to spread and disperse onto extracellular matrix molecules.

**Shh affects adhesion and dispersal of neural plate cells**

Because Shh can either be released in the cell’s environment or remain associated with the cell surface through coupling to lipid moities and binding to extracellular matrix molecules (Incardona and Eaton, 2000; Ingham, 2000; Ingham and McMahon, 2001; Ma et al., 2002), we tested its effect under the following different experimental conditions: it was applied directly in solution in the culture medium, in an immobilized form adsorbed onto the dish together with FN or LN1, or produced by neuroepithelial cells themselves after transfection. When tested in solution, Shh was used at concentrations ranging from 0.02 to 2 μg/ml. When immobilized onto the dish, the coating concentrations used were 10-fold greater, i.e., 0.2–20 μg/ml, because, using enzyme-linked immunosorbent assay (ELISA) and other biochemical and immunological assays, we could estimate that Shh adhered poorly to the plastic dish; at the coating concentration of 20 μg/ml, only 10% of the molecules remained effectively adsorbed onto the dish, and, at coating concentrations below 2 μg/ml, negligible quantities were adsorbed.

When Shh was added to the culture medium at the beginning of the culture, at all concentrations tested, the gross aspect of the explants as well as the morphology of individual cells appeared very similar to those observed in controls at least during the first 36 h in culture (Fig. 1E–H). In addition, after 48 h, at Shh concentrations greater than 0.2 μg/ml, a number of cells differentiated into neurons and extended neurites both in and out of the explants (Fig. 1G and H). In contrast, when Shh was applied in an immobilized form adsorbed onto the dish at coating concentrations above 10 μg/ml, the explants failed to flatten onto the substrate. Instead, during the first 24 h in culture, they adopted a compact shape and only a few cells were able to adhere to the dish (Fig. 1I and J). Video-microscopy analyses demonstrated that the shape of the explant did not evolve much during this time period (Fig. 2B). In the most extreme cases, the explants barely adhered to the dish and their apparent area shrunk during the first 24 h. The situation...
Fig. 3. Effect of Sonic hedgehog (Shh) overexpression on neuroepithelial cell adhesion and migration. Neural plates were transfected with cDNA constructs for alkaline phosphatase (AP) (A–C) or Shh (D–F) by in ovo electroporation, dissected out after 12 h of incubation, and subsequently cultured for 24 h on fibronectin (FN) substrates. (A and D) Comprehensive views of the explants showing rapid dispersion of neuroepithelial cells from control, AP-transfected explants (A), contrasting with the intense cell compaction and reduced cell dispersion in Shh-transfected explants (D). (B, C, E, and F) Corresponding GFP expression (B and E) and immunolabeling for Shh (C and F) in explants transfected for AP (B and C) and Shh (E and F). Arrows point at cells at the periphery of the explants. (G) Quantitation of the apparent areas of the transfected explants. Bar in A and D = 100 μm; bar in B, E, C, and F = 50 μm.
Fig. 4. Sonic hedgehog (Shh) affects integrin-mediated adhesion and migration of neuroepithelial cells. (A–D) Immunolabelings for the β1-integrin subunit in neural plate explants cultured for 24 h on fibronectin (FN) substrates (A and C) or on FN in the presence of Shh adsorbed onto the substrate at the coating concentration of 20 μg/ml (B and D). (A and B) Comprehensive view of the explants showing that expression of β1-integrins is not significantly affected in cells upon exposure to Shh. (C and D) Detailed view of the surface distribution of β1-integrins in cells situated at the periphery of the explants. Control cells exhibit a broad lamellipodium typical of motile cells. β1-integrins are concentrated in focal adhesion sites (short arrows) as well as at the periphery of the lamellipodium (arrowheads). In Shh-treated cells, in contrast, cell processes are retracted and do not form lamellipodia (long arrows). Integrins are redistributed over the cell surface and are not concentrated in focal adhesions. (E) Spreading of isolated neuroepithelial cells on FN and laminin-1 (LN1) with soluble or adsorbed Shh, as measured in cell adhesion assays. (F) Effects of Mn2+, Mg2+, and Ca2+ on neuroepithelial cell spreading on FN in the presence of Shh adsorbed to the substrate. Cell adhesion is restored almost entirely in the presence of Mn2+ at 1000 μM but not with Mg2+ or Ca2+. Bar in A and B = 100 μm; bar in C and D = 10 μm.
remained unchanged until the second day in culture, as further evidenced by the rather weak increase in the area of the explants (on adsorbed Shh, a 4-fold increase after 36 h compared to the 20-fold increase on FN and the 15-fold increase in the presence of Shh in solution; Fig. 2C). After 48 h, while the explants were highly compact, neuronal differentiation occurred with neurites often extending on the substrate (Fig. 1K and L).

To rule out possible artifacts due to a chase effect of Shh on the adsorption of the matrix molecules on the substrate, we quantified by immunoassays the relative amounts of FN or LN1 adsorbed to the dish. We found that the decrease in their amount adsorbed in the presence of Shh was limited even at high doses of Shh and could not account for the strong reduction of explant spreading, as it never exceeded 10–30% of the initial amount deposited. In addition, Shh effect on cell adhesion could not be attributed to simple obstruction of the adhesion sites of FN and LN1 substrates, as it could be specifically inhibited by antibodies to defined epitopes of the Shh molecule and that mutated forms of Shh were inactive in our assay (see below).

To further assess Shh effects on neuroepithelial cell adhesion and dispersion in a cellular context, neural plates were transfected using in ovo electroporation with a cDNA construct for Shh. Control experiments were carried out using a cDNA construct for alkaline phosphatase (AP). A GFP construct was coelectroporated with Shh or AP to allow tracing of transfected cells. Twelve hours after electroporation, neural plates were explanted in vitro on FN substrates and neuroepithelial cell dispersion was monitored during the subsequent 24 h by measuring the overall surface of the explants. Transfection efficiency was estimated at first by visualization of GFP-labeled cells and confirmed by immunocytochemistry for Shh or by revelation of AP activity. It was found that cells expressing detectable levels of GFP expressed systematically significant levels of Shh or strong AP activity (not shown). Neural plate explants transfected with AP showed the same propensity to disperse on FN as nontransfected explants (Fig. 3A). GFP-expressing cells were scattered both in the center and in the periphery of the explant (Fig. 3B). No Shh expression was found in neuroepithelial cells except in a few cells confined to the center of the cell mass, presumably corresponding to the floor plate (Fig. 3C). Explants electroporated with Shh and showing a high proportion of transfected cells were poorly flattened and instead showed a compact aspect with few spread cells at the periphery (Fig. 3D). Shh-expressing cells were essentially restricted to the compact cell mass (Fig. 3E and F). Interestingly, explants containing low proportions of transfected cells exhibited a normal, flattened aspect and dispersed rapidly. However, in those explants, Shh-expressing cells were systematically absent from the periphery. Quantification of the area occupied by the explants revealed that more than 60% of the explants transfected with Shh occupied a surface lower than 1 mm², whereas about 50% of the explants transfected with AP were at least twice as large (Fig. 3G).

Collectively, these data indicate that, when immobilized to the bottom of the dish with extracellular matrix molecules or expressed by neuroepithelial cells themselves, Shh can profoundly modify the adhesive properties of neural plate explants.

In the subsequent experiments, we next compared the biological activities of Shh applied in solution at 2 μg/ml and adsorbed onto the substrate at the coating concentration of 20 μg/ml.

**Shh effects on integrin- and cadherin-mediated adhesion of neuroepithelial cells**

Previous studies have established that neuroepithelial cells at the onset of neurulation express essentially β1-integrins as adhesion receptors for FN and LN1 and N-cadherin as cell–cell adhesion receptors (Duband et al., 1986, 1988). We then investigated whether Shh adsorbed onto the substrate affected expression and function of β1-integrin and N-cadherin.

Immunofluorescence labeling of neural plate explants confronted with Shh immobilized to the substrate revealed no major change in the overall expression of the β1-integrin subunit compared with controls or with explants treated with soluble Shh, at least during the first day in culture. All cells expressed conspicuous levels of integrins whatever their position within the explants (Fig. 4A and B). However, detailed analysis of the surface distribution of β1-integrins on cells situated at the periphery of the outgrowth showed major reorganizations of adhesion sites in Shh-treated cells, correlating with changes in cell morphology. While, in untreated motile cells, β1-integrins displayed their characteristic pattern both as a continuous belt at the periphery of a broad lamellipodium as well as in numerous focal contacts (Fig. 4C), cells confronted with Shh immobilized on the substrate were devoid of lamellipodia and, instead, showed long, thin cellular processes. β1-integrins were essentially diffuse over the cell surface and few focal contact sites could be visualized (Fig. 4D). This observation of β1-integrins being redistributed over the cell surface upon Shh treatment is indicative of a strong decrease in integrin activity. To quantify this, we measured the adhesion capacities of neuroepithelial cells in adhesion assays with isolated neural plate cells in suspension. As shown on Fig. 4E, cells spread efficiently over FN and LN1 substrates in the complete absence of Shh, consistent with their ability to disperse rapidly on these substrates. About 35–40% of the cells were spread after 1 h and maximal cell spreading of 50% was usually achieved after 2 h. In contrast, while soluble Shh added to the culture medium during the adhesion assay did not alter significantly the spreading capacities of neural plate cells, immobilized Shh abolished cell adhesion to FN and LN1 almost entirely, thereby confirming that integrin function was affected (Fig. 4E). To test whether Shh might
affect integrin affinity in neuroepithelial cells, adhesion assays were carried out in the presence of increasing concentrations of Mn$^{2+}$, known to induce highly active conformation of integrins. As shown on Fig. 4F, Mn$^{2+}$ at 1000 $\mu$M was able to counteract almost entirely the inhibitory effect of Shh on neuroepithelial cell adhesion while Mg$^{2+}$ and Ca$^{2+}$ were ineffective, suggesting that Shh could indeed abolish substrate adhesion by converting integrins toward an inactive state.

To determine whether neuroepithelial cell compaction induced by Shh was mediated via N-cadherin, neural plate explants were confronted with Shh adsorbed onto the substrate in the presence of antibodies to N-cadherin during the first 24 h in culture, i.e., at the time when compaction occurs. Blocking antibodies to N-cadherin did not significantly affect cell spreading and dispersion among control explants (Fig. 5A). The same antibodies, in contrast, induced rapid and extensive cell dissociation in explants confronted with Shh adsorbed on the substrate. After 12–24 h, the whole explant fell apart and individual cells were dispersed (Fig. 5B). It is noteworthy that cell spreading was not restored upon treatment with the antibodies, thus further confirming that inhibition of cell spreading by Shh did not result from increased cell compaction. Finally, nonblocking antibodies failed to affect both cell dispersion in control explants (Fig. 5C) and cell cohesion in explants confronted with Shh (Fig. 5D). These observations therefore indicate that Shh induced cell cohesion among neuroepithelial cells via N-cadherin. However, quantitation experiments using RT-PCR revealed that it did not modify the overall level of transcripts for N-cadherin (Fig. 5E).

**Dual role of Shh on neuroepithelial cell adhesion and differentiation**

Because Shh has been primarily identified as a differentiation-promoting factor in the neural epithelium, we analyzed whether, under our experimental conditions, it was able both to induce cell differentiation and to reduce cell dispersion. To this aim we monitored by immunostaining the appearance over time of a variety of cellular markers of ventral neuroepithelial cells in explants confronted or not with soluble or adsorbed Shh: Islet1/2 was selected as a marker for motor neurons, HNF3β as a marker for floor plate cells, and BEN as a marker for both cell types (Ericson et al., 1996; Pourquié et al., 1990). In the complete absence of exogenous Shh, virtually no cell in explants showed detectable levels of labeling for any of the markers during the first 48 h in culture and, after 72 h, only a few cells exhibited a faint staining for BEN (Fig. 6D), Islet1/2 (Fig. 6E), or HNF3β (Fig. 6F). In the presence of soluble Shh at concentrations higher than 0.2 $\mu$g/ml, significant proportions of cells started to express BEN on their surface, Islet1/2 or HNF3β in their nuclei after 2 days in culture, correlating with neurite outgrowth in the explant (see Fig. 1G). After 3 days, the number of BEN-, Islet1/2-, or HNF3β-positive cells became maximal (Fig. 6G–I). Quantitation of the BEN-positive cells during the time course of the culture confirmed that, in the presence of Shh, cell differentiation became obvious after 48 h and was complete after 72 h (Fig. 6M). In addition, the number of BEN-positive cells in neural plate explants increased with Shh concentration within the range of 0.2–2 $\mu$g/ml (Fig. 6N). Finally, we found that Shh was able to induce the formation of neurons with the same potency on FN and LN1 (Fig. 6N). Our observations are consistent with previous reports showing that soluble Shh induces differentiation of neuroepithelial cells into ventral spinal cord cells within 48 h in culture and in a dose-dependent manner (Roelink et al., 1995).

When adsorbed onto the substrate at coating concentrations greater than 10 $\mu$g/ml, Shh induced ventral cell type differentiation in neural plate explants in about the same proportions and with the same time course as soluble Shh (Fig. 6J–N). Interestingly, Shh caused cell compaction and reduced cell dispersion within the first day of culture prior to cell differentiation, therefore excluding the possibility that changes in the adhesive properties of cells observed in the presence of Shh might result from induction of differentiation. After 24 h, few cells exhibited detectable levels of labeling for the any of the markers, while being highly cohesive (Fig. 6A–C and M).

**Shh effects on neuroepithelial cell adhesion and differentiation involve distinct cellular mechanisms**

Because Shh was able to induce a dual adhesion and differentiation response in the same cell population in the neural plate, it was therefore of interest to analyze whether those responses involved distinct cellular processes. For this purpose, we analyzed the activity of mutated constructs of the Shh protein and blocking antibodies directed against different epitopes of the molecule. The molecular characteristics and the biological activities of these mutated forms of Shh and of the antibodies were described elsewhere (Testaz et al., 2001; Williams et al., 1999). In addition, using a variety of immunological and biochemical assays, we verified that these mutated forms bind to the plastic dish with the same efficacy as wild-type Shh.

The Shh(polyQ) mutant, which is not able to bind the Patched receptor, failed to induce both neuronal cell differentiation and compaction of neural plate explants. After 72 h of culture, these explants presented the same flat morphology as controls (Fig. 7A), and no neuritic sprouting was observed whether the Shh(polyQ) mutant was applied in solution in the medium or adsorbed onto the dish. In contrast, the Shh(N-9/C-3) mutant, which binds Patched with the same apparent affinity as wild-type Shh, promoted neuronal differentiation but did not affect cell adhesion and dispersal among the explant (Fig. 7B). Quantification of BEN-expressing cells (Fig. 8A) and cell spreading assays (Fig. 8B) confirmed that the Shh(polyQ) mutant was inactive in the control of both differentiation and adhesion of
neural plate cells, while the Shh(N-9/C-3) was as potent as wild-type Shh to promote cell differentiation but affected spreading of neuroepithelial cells only slightly. As described by others (Ericson et al., 1996), the mAb 5E1 used as a competitor for Shh totally abolished Shh activity on neuronal differentiation (Fig. 8A) but was unable to block its effect on spreading and dispersion of neuroepithelial cells (Fig. 7C). The mAb ASC1, in contrast, whose binding site maps to a different domain of the Shh molecule (Testaz et al., 2001), strongly affected Shh function as it prevented both neuron differentiation and compaction of neural plate explants (Figs. 7D and 8A).

We also investigated the signaling pathways involved in the control of neuroepithelial cell adhesion and differentiation using compounds known to interfere specifically with downstream targets of Shh signals. Consistent with a previous report (Incardona et al., 2000), cyclopamine, a plant steroidal alkaloid that has been found to inhibit Shh activity (Taipale et al., 2000), totally abrogated neurite extension and differentiation of BEN-positive cells in neural plate explants (Figs. 7E and 8C). However, it was poorly effective in reversing Shh effect on compaction of neural plate explants (Fig. 7E) and on the reduction of the initial spreading of isolated neuroepithelial cells (Fig. 8D). Likewise, forskolin, another inhibitor of Shh signals (Ericson et al., 1996), was able to inhibit cell differentiation induced by soluble or adsorbed Shh as well as by the Shh(N-9/C-3) mutant (Figs. 7F and 8C), but failed to affect the explant compaction and to restore cell spreading induced by adsorbed Shh (Figs. 7F

Fig. 5. Explant compaction induced upon Sonic hedgehog (Shh) exposure is mediated by N-cadherin. (A and D) Overall views of neural plate explants cultured for 12 h on fibronectin (FN) substrates without (A and C) or with Shh adsorbed onto the substrate at the coating concentration of 20 µg/ml (B and D) and in the presence of blocking (A and B) or non-blocking (C and D) antibodies to N-cadherin. (E) Reverse transcriptase-polymerase chain reaction analysis of N-cadherin expression in neural plate explants confronted or not with soluble or adsorbed Shh. Bar = 100 µm.
Fig. 6. Dual role of Sonic hedgehog (Shh) on neuroepithelial cell adhesion and differentiation. (A–L) Immunofluorescence labeling for BEN (A, D, G, and J), Islet1/2 (B, E, H, and K), and HNF3β (C, F, I, and L) in explants cultured for 24 h on fibronectin (FN) with Shh adsorbed on the substrate (A–C) and for 72 h on FN (D–F), FN in the presence of soluble Shh (G–I), or FN in the presence of Shh adsorbed to the substrate (J–L). (M) Proportion of BEN-positive cells in neural plate explants with time of culture on FN with soluble or adsorbed Shh. (N) Proportion of BEN-positive cells in neural plate explants cultured for 3 days on FN or laminin-1 (LN1) with increasing concentrations of soluble or adsorbed Shh. Bar in A = 100 μm; bar in D = 200 μm.
and 8D). In addition, neither cyclopamine nor forskolin affected significantly adhesion of neuroepithelial cells when added to the culture medium in the absence of Shh (Fig. 8D).

Taken together, these experiments confirm and extend our previous data (Testaz et al., 2001) and show that Shh can both promote ventral cell type differentiation and reduce cell-substrate adhesion on the same cell population through distinct cellular events. While the classical Patched-Smoothed-Gli pathway could be implicated in the Shh control of cell differentiation, inhibition of cell spreading and induction of compaction appeared independent of this pathway.

Discussion

In a previous study, we have shown that Shh is able to control neural crest cell dispersion from the dorsal part of the neural tube by inhibiting their integrin-mediated adhesiveness (Testaz et al., 2001). In the present article, we provide evidence that Shh exerts a dual activity on adhesion and differentiation of early neuroepithelial cells. Indeed, presenting Shh protein in an immobilized form in association with the extracellular matrix or produced by the cells not only induces massive cell differentiation into floor-plate cells and motoneurons in the neural plate but also results in an important compaction of the explants associated with a
dramatic reduction in their adhesion to the substrate. Moreover, Shh regulation of adhesion properties during neural tube morphogenesis is rapid and reversible, it does not involve the classical Patched-Smoothened-Gli signaling pathway, and it is independent and discernible from Shh-mediated cell differentiation. Thus, modifications of the adhesive properties of neural epithelial cells induced by Shh cannot be attributed to its differentiation-promoting effect but reveal a novel function of Shh in this tissue that has never been described before.

Shh regulation of cell adhesion may contribute to early neural tube morphogenesis

In a recent report, Ybot-Gonzales et al. (2002) have reinvestigated the process of neural tube closure in the mouse embryo and have provided evidence that bending of the neural epithelium in its midline and dorsolateral portions is regulated by mutually antagonistic signals derived from the notochord and surface ectoderm and possibly involving Shh and BMPs. The molecular mechanisms involved in this process are not known yet, but it is likely that changes in cell adhesion are critical events during progression of neurulation. Perturbations of cell interactions and of cytoskeletal organization induce major morphological defects in the neural tube, among which absence of neural tube closure is the most commonly found (Bronner-Fraser et al., 1992; Brouns et al., 2000; Hildebrand and Soriano, 1999; Kil et al., 1996). In addition, it has already been reported that members of the transforming growth factor-β family are able to regulate the activity and/or expression of adhesion molecules of neural epithelial cells, particularly at the time of neural crest cell migration (Delannet and Duband, 1992; Sela-Donenfeld and Kalcheim, 1999).

Our present observations show that neural plate cells at the time of early neurulation in the trunk are readily able to...
spread and to disperse to form a monolayer of undifferentiated neuroepithelial cells when explanted in vitro onto extracellular matrices. However, at later stages of neurulation, i.e., after neural tube closure, with the exception of the prospective neural crest, these cells are no longer able to dissociate and disperse, instead they remain as a compact structure on the substrate (Delannet and Duband, 1992; Newgreen and Minichiello, 1996). These changes in the ability of neuroepithelial cells to disperse in vitro are believed to reflect gradual changes in their cell-cell and cell-substrate adhesion properties during progression of neurulation in vivo. That addition of Shh can prematurely reduce the ability of neural plate cells to disperse in vitro and that this effect can be achieved prior to induction of cell differentiation indicates that a primary role of Shh during neurulation would be to control cell adhesion during morphogenetic movements in the neural tube. This view is further supported by the recent demonstration of the progressive ventro-dorsal expansion of Shh protein during neural tube closure (Gritli-Linde et al., 2001).

**Neuroepithelial cells respond to Shh effect on cell adhesion in a similar manner to neural crest cells**

Our data indicate that the effect of Shh on the dispersal of neural plate cells can be essentially attributed to the reduction of the integrin-mediated substrate-adhesion properties of the cells. Thus, Shh effect on integrin function is not restricted to neural crest cells (Testaz et al., 2001) but also applies to other embryonic cells, thereby raising the intriguing possibility that Shh might affect cell-substrate adhesion during a large variety of morphological events throughout the course of embryonic development. While the integrin repertoire expressed by neural crest cells has been thoroughly documented in vitro (Delannet et al., 1994; Desban and Duband, 1997; Testaz et al., 1999), relatively little is known about the expression pattern of integrins during neural tube morphogenesis in the avian embryo. So far, only the β1 and α1 subunits have been detected in neuroepithelial cells at the neural plate stage (Duband et al., 1986, 1992; Krotoski et al., 1986), corresponding to a strong accumulation of both FN and LN1 at the basal side of the neural epithelium (Duband and Thierry, 1982, 1987). Injections of antisense oligonucleotides against the β1, α1, α4, and α5 subunits have been shown to cause important neural tube abnormalities (Kil et al., 1996), emphasizing a prominent role of FN and LN1 interactions with α1β1, α4β1, and α5β1-integrins during neurulation. In complete agreement with these previous studies, our data indicate that neural plate cells adhere efficiently to FN and LN1, and that Shh inhibits neuroepithelial cell spreading to these substrates.

A striking feature of Shh activity on neural plates cultured in vitro is that, in addition to reducing explant adhesion, it also caused its strong compaction. Our data indicate that such a compaction is mediated by N-cadherin but that it is not accompanied by an increase in the corresponding messengers, suggesting that it involves merely stabilization of preexisting cell-cell junctions. Whether Shh is directly responsible for these modifications is not known yet. It is noteworthy that cell-cell and cell-substrate adhesion systems are tightly interconnected events and it cannot be excluded that Shh induced explant compaction as a consequence of the inhibition of substrate adhesion. Indeed, interfering with integrin activity in neural crest cells has been shown to inhibit cell migration and to cause a rapid N-cadherin-mediated cell clustering (Monier-Gavelle and Duband, 1997). However, in favor of a possible direct control of Shh on cell-cell adhesion are the observations that N-cadherin expression occurs at first in the ventral midline of the neural plate and expands gradually toward the dorsolateral sides of the neural plate with a time course that mirrors the distribution of Shh signals (Aaku-Saraste et al., 1996; Duband et al., 1988).

**Shh effect on neuroepithelial cell adhesion involves a nonconventional mechanism**

In the present study, we describe a novel function of Shh that differs strikingly by its characteristics from the other reported Shh activities, e.g., promotion of cell differentiation; it is immediate and it does not involve the canonical Patched-Smoothened-Gli signaling pathway. In addition, to be effective on neuroepithelial cell adhesion, Shh must be presented in an immobilized form in association with the extracellular matrix. However, it should be stressed that this mode of presentation of Shh to the cell does not preclude its other functions, as it does not alter its potency to induce ventral cell-type differentiation, nor does it change the signaling cascade involved. Reduction of dispersal and compaction of neural plate cells were also achieved when Shh was produced by neuroepithelial cells themselves, therefore ruling out any possible artifact due to the adsorption of the Shh molecule to the culture dish. Interestingly, only Shh-expressing cells exhibited striking modifications in their adhesive capacities, indicating that this effect is essentially local and does not operate at distance.

The incidence of the mode of presentation of Shh on its biological activities may be reflected by the biochemical specificities of the Shh molecule that influence its interactions with the cell surface, and as a consequence, its signaling activity. Indeed, Shh has been found to undergo post-translational modifications, including addition of palmitate and cholesterol moities at the N- and C-termini of the molecule, known to result in the restriction of Shh to the cell membrane, thereby increasing its local concentration and greatly enhancing its potency (Khotz et al., 2001; Pepinsky et al., 1998; Porter et al., 1996; Zeng et al., 2001). Such lipid-modified forms of the Shh protein may be mimicked by adsorbing Shh onto the substrate and are also naturally encountered in cells transfected with full-length Shh constructs. Interestingly, we found that these N- and C-terminus sequences of Shh that undergo lipid modifications are re-
quired for its effect on cell adhesion but are dispensable for the induction of cell differentiation. Moreover, at least the N-terminus sequence is highly conserved among the different members of the hedgehog family throughout evolution, although it is apparently not involved in binding to the Patched receptor, indicating that it may be critical for the biological functions of the molecule. On the other hand, hedgehog proteins are able to bind a variety of extracellular matrix components and these interactions have been shown to affect their functions. For example, during development of the cerebellum, Shh interacts with heparan-sulfate proteoglycans, and this interaction is necessary to achieve maximal proliferation of mature granule cells (Rubin et al., 2001). Likewise, granule cell precursors grown in vitro on LN1 display a proliferative response to Shh, while when plated on FN, they show enhanced neuronal differentiation at the expense of proliferation (Pons et al., 2001).

Integrating the dual Shh activities during neurulation

In our previous studies, we have shown that Shh modulates substrate adhesion of neural crest cells without perturbing their proliferation and differentiation (Testaz et al., 2001). In the present study, we provide evidence that Shh can affect both adhesion and differentiation within the same cell population, but in a sequential manner, therefore raising the important question of the connections between these two events. Preliminary data from our laboratory tend to suggest that Shh activity on cell differentiation is not potentiated by adhesion events. Preliminary data from our laboratory tend to suggest that Shh activity on cell differentiation is not potentiated by its effect on cell adhesion, therefore arguing in favor of a complete independence of adhesion and differentiation events regulated by Shh. However, it has been found that, once cell differentiation is in process, adhesion properties of cells are irreversibly modified (Aaku-Saraste et al., 1996). Thus, it can be proposed that Shh-mediated regulation of adhesion in neuroepithelial cells is a two-step event: at first, a direct, immediate, and short-term effect on integrin and cadherin functions, essentially involving posttranscriptional events, and later, an indirect, long-term effect on the repertoire of cadherin and integrins, possibly at the transcriptional level, and resulting from neuronal differentiation.

In conclusion, the present report confirms and extends our previous studies and shows that Shh can regulate sequentially adhesion and differentiation events within the same cell population during neural tube morphogenesis. Recent studies have shed light on novel functions of Shh during migratory processes in developing embryos. In Droso-

 either by the canonical Patched-Smoothened-Gli signaling pathway or by alternative mechanisms, thereby revealing the diversity of cellular responses to hedgehog signals. Thus, Shh may regulate sequentially and coordinate multiple events during early neurulation in vertebrate, i.e., cell adhesion, survival, proliferation, differentiation, and axon guidance. The integration of the long-range ventro-dorsal Shh signal with a regional-dependent extracellular matrix signal would allow neural plate cells to develop several responses to Shh protein, differing by their nature, timing, and chronology. Moreover, the interactions between the different morphogens, as, for example, the opposed activities of BMPs and Shh, as well as their fine regulation by the interplay of specific antagonists, such as Hip and Gas1 for Shh, and noggin, chordin, and follistatin for the BMPs, would introduce another level of control by which a limited set of morphogens are able to regulate the different aspects of neural plate cell behavior to progressively form the future spinal cord.

Acknowledgments

We thank B. Gafield, T. Jaffredo, and K. Yamada for their gifts of reagents. This study was supported by the CNRS, the Université P. et M. Curie, ARC (9998 and 4260), the Association Française contre les Myopathies, and the Fondation pour la Recherche Médicale. A.J. is a recipient of a doctoral fellowship from the Ministère de l’Education Nationale. This article is dedicated to the memory of André Adoutte.

References


