β-Catenin signaling initiates the activation of astrocytes and its dysregulation contributes to the pathogenesis of astrocytomas

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Abstract

Astrocytes are the most abundant cell of the CNS and demonstrate contact inhibition in which a nonproliferative, nonmotile cellular state is achieved once stable intercellular contacts are formed between mature cells. Cellular injury disrupts these intercellular contacts, causing a loss of contact inhibition and the rapid initiation of healing. Dysregulation of the molecular pathways involved in this process is thought to lead to an aggressive cellular state associated with neoplasia. We investigated whether a comparable correlation exists between the response of astrocytes to injury and the malignant phenotype of astrocytomas. We discovered that the loss of contact inhibition plays a critical role in the initiation and regulation of reactive astrocytes in the healing of wounds. In particular, injury of the astrocytes interrupts and destabilizes the cadherin-catenin complexes at the cell membrane leading to nuclear translocation of β-catenin and characteristic changes associated with the activation of astrocytes. Similar signaling pathways are found to be active—but dysregulated—in astrocytomas. Inhibition of β-catenin signaling diminished both the response of astrocytes to injury and induction of the malignant phenotype of astrocytomas. The findings shed light on a unique mechanism associated with the pathogenesis of astrocytomas and provide a model for understanding the mechanisms of tissue repair and tumorigenesis in the brain.

Results

Spatial and Temporal Changes in Reactive Astrocytes. Current models of the activation of astrocytes include morphological, migratory, and molecular changes. Other key features of this process include growth and proliferative changes, differentiation of quiescent astrocytes, and an increase in the expression of cell biomarkers. An in vitro scratch assay for the healing of wounds in astrocytomas was performed. Wounds were observed at defined time points to determine the morphological changes that occur during the activation of astrocytes (Fig. L4). This assay demonstrated the migration of astrocytes into the wound within 1 h of injury and complete infiltration by 6 d postinjury. High-frequency serial images of astrocytes were captured following injury to observe these changes in greater detail. Cells in the region adjacent to injury developed either a migratory phenotype, with processes extending into the cavity of the wound, or a proliferative phenotype along the margins of the wound. Cells farthest from the margins remained quiescent (Movies S1, S2, and S3).

Immunofluorescence staining was performed to identify a spatial and temporal pattern of expression of molecular markers associated with the activation of astrocytes. Following injury, the expression of both glial fibrillary acidic protein (GFAP) and Nestin increased at the margins of the wound. GFAP was strongly expressed in migratory cells in the cavity of the wound but cells expressing Nestin were localized at the margins of the wound and the immediately adjacent region (Fig. L8). Expression of several markers associated with growth and proliferation of astrocytes [nuclear receptor corepressor (NcoR), ciliary neurotrophic factor receptor (CNTFR)], the differentiation of astrocytes (GFAP, vimentin), and stem and progenitor cells [Nestin, sex-determining region Y-box 2 (SOX2)] also increased following injury (Fig. L1C).

The expression of these markers followed a distinct spatial localization and pattern of separation at the site of injury. The expression of SOX2 was increased at the margins of the wound, but other potential stem cell biomarkers, including Nanog and Oct4, were not increased following injury to the astrocytes (Fig. S1). We then determined the time course of expression of several markers in the astrocytes that were up-regulated in response to injury. Within a day after injury, several markers, including NcoR, epidermal growth factor receptor (EGFR), GFAP, and SOX2 had increased. The expression of Nestin increased transiently, peaking at 3 d postinjury, and the expression of SOX2...
that rapidly sensed mechanical injury. The expression of β-catenin before and after injury was measured to determine whether it was directly involved in the activation of astrocytes. The expression of β-catenin was increased at 1 and 4 h following injury. Specifically, there was up-regulation of β-catenin phosphorylated at serine 675 (Ser-675) (Fig. 2B). Western blot analysis of astrocytes after injury demonstrated an increase in both total and Ser675-phosphorylated β-catenin (Fig. 2C and Fig. S3).

Increased Ser-675-phosphorylated β-catenin in response to injury suggests a mechanism where β-catenin dissociates from membrane complexes after phosphorylation, accumulates in the cytoplasm, and translocates into the nucleus. Immunoprecipitation was performed to investigate this mechanism by measuring β-catenin binding to various cadherins at the membrane surface following injury. β-catenin was found to have decreased association with N-cadherin, an important mediator of cellular adhesion in the nervous system (9) (Fig. 2D and Fig. S4). Because these findings are suggestive of a process in which dissociation of β-catenin from the cell membrane occurs, we investigated whether translocation of β-catenin into the nucleus also occurs during the activation of astrocytes by measuring downstream targets within the lymphoid enhancer factor (LEF) and T-cell factor (TCF) families of transcription factors (10). Expression levels of LEF1 and TCF4 were up-regulated 6 h following injury, suggesting an increase in the nuclear effects of β-catenin (Fig. 2E and Figs. S5 and S6).

Expression of β-catenin in membrane-bound, cytoplasmic, and nuclear cell fractions following injury was measured to determine how the expression of β-catenin increases in response to injury. Cytoplasmic and nuclear expression of β-catenin increased following injury, but membrane-bound β-catenin is decreased shortly after injury (Fig. 2F and Fig. S7). The findings indicate that injury of astrocytes causes destabilization and phosphorylation of β-catenin at the cell membrane, translocation to the nucleus, and the initiation of activation of astrocytes.

**Inactivation of β-Catenin Diminishes the Reactive Astrocyte Phenotype.**

siRNA knockdown of β-catenin was performed and the expression of various markers previously found to be associated with this process was measured to further elucidate the role of β-catenin in the activation astrocytes (Figs. S8 and S9). Following injury, knockdown of β-catenin resulted in minimally decreased expression of GFAP, but significantly decreased expression of CNTFR at the margin of the wound. Additionally, expression of the stem cell markers Nestin and SOX2 was nearly abolished by the knockdown of β-catenin (Fig. 3A). The findings indicate an important role of β-catenin in the proliferative capacity of cells and the expression of stem-cell markers during the activation of astrocytes.

The ability of astrocytes to repair after injury before and after the knockdown of β-catenin was measured to determine whether astrocyte repair of injury is affected by the loss of β-catenin. The studies revealed that inhibition of β-catenin had a detrimental effect on the repair of astrocytes (Fig. 2B). Additionally, the use of an inhibitor of glycolgen synthase kinase-3β (GSK3β), a kinase that increases the degradation of β-catenin, resulted in faster healing of the wound (Fig. 3C). These results suggest that β-catenin is a critical mediator of the activation of astrocytes and that a reduction of its function results in a diminished response to injury by astrocytes.

**Dysregulation of Reactive Astrocyte Signaling Mediated by β-Catenin Plays a Role in the Pathogenesis of Astrocytomas.**

Previous studies have implicated β-catenin signaling and its nuclear translocation in the malignant phenotype of astrocytomas and demonstrated Wnt and the overexpression of β-catenin in these tumors (11–13). The finding that β-catenin signaling and nuclear translocation plays an important role in the activation and proliferation of astrocytes in response to injury led us to examine whether this signaling pathway might be common to these two processes (repair and tumorigenesis). To measure and confirm the expression of β-catenin in astrocytomas, an immunohistochemical and Western blot analysis was conducted on multiple
Cell-cycle analysis was carried out on the primary glioma cell lines to further examine their proliferative capacity. The cell demonstrated decreased S-phase population after knockdown of β-catenin, suggesting cell-cycle arrest and diminished proliferation as a result of the inhibition of this pathway (Fig. 5A). Additionally, using a sphere-formation assay, tumor sphere formation was noticeably reduced following the knockdown of β-catenin and spheres that were able to form in culture were of decreased diameter (Fig. 5B). Immunofluorescence analysis of various markers known to be involved in activation of astrocytes and the pathogenesis of astrocytomas confirmed that expression of β-catenin was strongly inhibited with siRNA transfection, and that c-myc, a downstream target of β-catenin, was also reduced (Fig. 5C and Fig. S11).

The expression of putative glioma stem cell biomarkers was measured before and after knockdown of β-catenin to determine whether β-catenin signaling plays a role in conferring or maintaining the characteristics of glioma cells. The expression of Nestin and SOX2 were dramatically decreased in primary glioma cell lines following transfection with either of the two siRNA constructs for β-catenin (Fig. 5D). These findings demonstrate that β-catenin signaling plays a significant role in the proliferative capacity, viability, and maintenance of primary glioma cells. Thus, β-catenin signaling may be a critical cellular pathway involved in the activation of astrocytes and its dysregulation may contribute to the pathogenesis of astrocytomas.

**Discussion**

Destabilization of the cell membrane plays a critical role in the initial sensing of mechanical injury and in the activation of astrocytes. Astrocytes respond in a defined way following injury that includes changes in migration, proliferation, and cell differentiation that contribute to the repair of injured tissue in the CNS. These changes occur rapidly. Migration and proliferation of astrocytes in the region of injury are apparent within 15 min following the in vitro scratch assay (Fig. S1). Similarly, the response of astrocytes to injury is briskly terminated once repair is

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**Fig. 2.** β-Catenin phosphorylation and nuclear translocation following the injury of astrocytes. (A) Quantitative PCR analysis quantifying the change in expression of various genes associated with cell adhesion following injury. Arrows indicate the expression of cadherin and β-catenin. (B) Immunofluorescence staining for phospho-β-catenin in Ser-675 (Top) and total β-catenin (Middle), 1 and 4 h postinjury. Following the injury of astrocytes, β-catenin is present in the nuclei of cells (Bottom). (Magnification: Upper, 200×; Lower, 400×.) (C) Western blot analysis demonstrating increased expression of total β-catenin and phospho-β-catenin at the Ser-675 site and decreased expression of phospho-β-catenin at other residues following injury. (D) Immunoprecipitation (IP) of N-cadherin and VE-cadherin and immunoblotting (IB) for β-catenin demonstrating decreased association of both N-cadherin and VE-cadherin with β-catenin following injury. (E) Western blot analysis demonstrating increased expression of downstream targets of β-catenin, LEF1, and TCF4, following injury. (F) Western blot analysis demonstrating expression of β-catenin in various cellular fractions at various time points following injury.
Changes associated with reactive astrocytes in astrocytomas of varying grade. (A) Tissue microarray analysis of β-catenin expression in astrocytomas of varying grade demonstrating a positive correlation of expression of β-catenin with the grade of the astrocytoma. (Magnification: 40x.) (B) Semiquantitative analysis of expression of β-catenin in samples of normal brain and tumors (individual bars) also demonstrating a positive correlation between expression of β-catenin and the grade of the astrocytoma. (C) Immunofluorescence staining for GFAP (red) and Nestin (green) demonstrating increased β-catenin in astrocytomas of increasing grade. (Magnification: 200x.)

β-Catenin signaling occurs through two pathways, one involving cadherin-catenin complexes at the cell membrane and the other occurring through Wnt/Frizzled signaling (18, 19). During embryological development, Wnt plays an important role in the patterning of cells in the nervous system and the regulation of development (20). Binding of Wnt to receptors of the Frizzled family leads to downstream evasion by β-catenin from degradation in the cytoplasm (21). The accumulation of β-catenin in the cytoplasm results in its entry into the nucleus and the transcription of various genes, including the LEF1/TCF family of transcription factors (22). In contrast to Wnt-dominated β-catenin signaling in embryogenesis, membrane cadherin-catenin complexes are responsible for both cell adhesion and contact inhibition of growth between astrocytes in adulthood. When cell-to-cell interactions are compromised by injury, or when activation of cell membrane-associated kinases, such as src or EGFR occurs, phosphorylation of β-catenin is thought to disrupt its interaction with constituents of the membrane complex, such as cadherins, α-catenin, and p120. As a result, increased cytoplasmic and nuclear β-catenin increases gene transcription and proliferation (23).
Cell-to-cell adhesion and normal tissue architecture are compromised after mechanical injury to quiescent astrocytes. Based on the current observations, it appears that translocation of β-catenin to the nucleus plays a critical role in propagating the changes associated with the activation of astrocytes (Fig. 6). The induction of this molecular cascade following injury to the astrocytes produces three populations of astrocytes, each of which contributes to the changes in proliferation, migration, and differentiation associated with the activation of astrocytes. One group is comprised of reactive migratory cells characterized by inwardly growing pseudopodia and processes into the cavity of the wound. These cells are distinguished by changes involving their differentiation and activation, particularly their increased expression of GFAP, a well-recognized marker of reactive astrocytes (Fig. 1B). A separate group of astrocytes consists of reactive proliferating cells with stem-cell-like properties that are characterized by their localization to the margins and immediate submarginal regions of the wound. Their expression of Nestin and SOX2 biomarkers are thought to identify neural stem and progenitor cells within the nervous system (24, 25). A third group of astrocytes is the quiescent cell population located farthest from the wound, with no identifiable change in response to injury. Once repair is complete, reactive astrocytes revert to this quiescent state in which GFAP, Nestin, and other expression markers associated with activation of astrocytes are no longer detectable.

Given the critical role of β-catenin signaling in the activation of astrocytes, this molecular pathway could also be implicated in the pathogenesis of astrocytomas. Overexpression of β-catenin in gliomas is well recognized and present observations confirm a positive correlation between the expression of β-catenin and the histological grade of astrocytomas (26) (Fig. 4). Our observations that specific downstream targets of β-catenin signaling, LEF1 and TCF4, are up-regulated in astrocytes following injury, and that β-catenin accumulates in the nucleus during the activation of astrocytes as well as in astrocytomas (13), suggest a common pathway between injury repair and tumorigenesis. The expression of neural stem cell markers Nestin and SOX2 in certain reactive astrocytes and in primary glioma cultures also suggests that similar molecular mechanisms contribute to the activation of astrocytes and pathogenesis of astrocytomas (27, 28). Treatment of primary glioma cell lines with β-catenin siRNA resulted in a markedly decreased potential for tumor sphere formation and the proliferative capacity of these cells (Fig. 5A and B). The expression of putative glioma stem cell markers was strongly inhibited after the knockdown of β-catenin (Fig. 5C and D). These findings indicated that the malignant phenotype of astrocytomas is at least partially regulated by β-catenin signaling and that the molecular mechanisms underly the activation of astrocytes are closely related to the mechanisms of pathogenesis and the progression of growth of astrocytomas. Patients with Turcot syndrome highlight the involvement of β-catenin signaling in tumorigenesis within the CNS, where mutations of the APC gene, an important regulator of β-catenin signaling, cause the formation of brain tumors (29).

Following the injury of astrocytes, distinct populations of reactive astrocytes express either GFAP or Nestin and localize to the margins of the wound with a consistent and replicable pattern in vitro, in which proliferating cells are initially found at the margins of the wound and motile, differentiated reactive proliferating cells with stem-cell-like properties that are characterized by their localization to the margins and immediate submarginal regions of the wound. Their expression of Nestin and SOX2 biomarkers are thought to identify neural stem and progenitor cells within the nervous system (24, 25). A third group of astrocytes is the quiescent cell population located farthest from the wound, with no identifiable change in response to injury. Once repair is complete, reactive astrocytes revert to this quiescent state in which GFAP, Nestin, and other expression markers associated with activation of astrocytes are no longer detectable.

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pattern (Fig. 1B). Expression of both GFAP and Nestin were also shown to correlate positively with increasing grade of astrocytomas. Cells expressing these markers minimally colocalized with each other and were dispersed in a heterogeneous manner in gliomas of increasing grade (Fig. 4C). The correlation between histological grade of tumors and the increasingly “reactive” nature of higher-grade astrocytomas provides evidence that dysregulation of the mechanisms involved in the normal activation of astrocytes could contribute to the pathogenesis of astrocytomas. Specifically, dysregulation of normal β-catenin signaling pathways involved in the activation of astrocytes could result in the functional loss of contact inhibition and an invasive, migratory phenotype observed in neoplastic states (50).

The time course of the activation of astrocytes have been characterized both morphologically and molecularly (Fig. 1 A and D). The dysregulation of this signaling pathway in astrocytomas appears to produce a desynchronized biological growth pattern in which the normal patterns of reactive astrocytes are lost and properties, such as proliferation and migration, occur in a chaotic, unregulated manner where GFAP (−), GFAP (+), and Nestin and Sox2 (+) cells are found throughout the chaotic 3D milieu of an astrocytoma. The potential loss of the dynamic regulation of the activation of astrocytes seen in normal injury compared with the dysregulated state of astrocytomas suggests a potential explanation for the dysregulated microenvironment of these tumors that appears to accelerate as tumors increase in grade. Although the association between CNS injury and tumor predisposition requires further investigation, genetic insult to the critical pathways involved in the activation of astrocytes demonstrated in this study could create a dysregulated state that results in the formation of brain tumors.

Materials and Methods

Tissue Culture. Primary human and mouse astrocyte tissue cultures were purchased and maintained according to the manufacturer’s protocol (ScienCell Research Laboratories). An in vitro scratch assay was used for the in vitro culture of astrocytes, as previously described (31–33). Monolayer cells were scraped with a sterile pipette tip. Cells were then incubated in fresh growth medium. Cellular morphology and healing of the wound were subsequently measured and recorded.

Primary glioma cell cultures were maintained under conditions described previously (34, 35). Cultures were dissociated into a single-cell suspension using Trypsin-Express (Invitrogen) for transfection and assayed for the formation of clones. Cells were resuspended at 50,000 cells/mL in DMEM/F12 supplemented with 50 ng/mL EFG, 20 ng/mL bFGF, and B27 (Invitrogen).

Glioma Tumor Samples and Tissue Dissection. Tissue samples were collected at the Cleveland Clinic Foundation. All tissue samples and clinical information were obtained as part of an Institutional Review Board–approved study for molecular analysis on brain tumor in Cleveland Clinic Foundation. Frozen tissue samples included grade II astrocytomas (n = 7), grade III astrocytomas (n = 2), and normal brain (n = 1). Tissue dissection was performed as previously described (36).

Paraffin-embedded brain tumor and normal tissue array (US Biomax) were used for immunohistochemistry staining. Normal brain tissue (n = 10), grade II glioma (n = 10), grade III glioma (n = 7), grade IV glioma (n = 9), and medullablastoma (n = 10) specimens were stained for β-catenin. Protein expression was measured by ImageJ software.

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