Dexamethasone Destabilizes Nmyc to Inhibit the Growth of Hedgehog-Associated Medulloblastoma

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Abstract

Mouse studies indicate that the synthetic glucocorticoid dexamethasone (Dex) impairs the proliferation of granule neuron precursors in the cerebellum, which are transformed to medulloblastoma by activation of Sonic hedgehog (Shh) signaling. Here, we show that Dex treatment also inhibits Shh-induced tumor growth, enhancing the survival of tumor-prone transgenic mice. We found that Nmyc was specifically required in granule cells for Shh-induced tumorigenesis and that Dex acted to reduce Nmyc protein levels. Moreover, we found that Dex-induced destabilization of Nmyc is mediated by activation of glycogen synthase kinase 3β, which targets Nmyc for proteasomal degradation. Together, our findings show that Dex antagonizes Shh signaling downstream of Smothened in medulloblastoma. Cancer Res 70(13): 5220–5. ©2010 AACR.

Introduction

Medulloblastoma is the most common malignant brain tumor in childhood. Despite significant advances in treatment strategies, many children die from this tumor due to the lack of therapeutic options that specifically target tumor-initiating and tumor-propagating mechanisms (1). One mechanism known to drive the growth of medulloblastoma in about 25% of human cases is constitutive activation of the Sonic hedgehog-Smothened (Shh-Smo) signaling pathway in cerebellar granule neuron precursors (CGNP; refs. 2, 3). Whereas in a normal situation, Shh signaling is only active in a precisely defined period of late embryonic and early postnatal cerebellar development, mutations in the Hedgehog pathway suppressor Patched or alterations of other pathway components result in permanent pathway activation and tumor formation (2, 4).

Both in mice and humans, these tumors display expression of the transcription factor Nmyc (3, 5), the stabilization of which prohibits cell cycle exit in normal development and medulloblastoma (5, 6). Inhibition of Hedgehog signaling by small molecule drugs has proved to effectively block the growth of medulloblastoma in mice (7), but significant side effects have been noted with some inhibitors (8).

We have previously shown that treatment with dexamethasone (Dex), a clinically used synthetic glucocorticoid, is able to inhibit Shh-induced CGNP proliferation both in vitro and in vivo at early neonatal stages (P0–P7) of cerebellar development (9). Now, we show that Dex treatment in P21 tumor-prone mice inhibits the growth of Shh-Smo–driven medulloblastoma and significantly prolongs survival. Genetic deletion of Nmyc in Math1-cre:SmoM2–associated medulloblastoma similarly resulted in inhibited tumor growth and prolonged survival. We show that Dex treatment results in decreased Nmyc protein levels and is associated with increased activity of glycogen synthase kinase 3β (GSK3β), which is known to promote destabilization through terminal phosphorylation of Nmyc in the MB1 domain (10).

Materials and Methods

Animals and animal treatment

All mouse procedures were done in accordance with National Research Council recommendations. SmoM2<sup>E1F1</sup> (Gt(Rosa)26Sor<sup>m1(Smo<sup>EYFP</sup>/Amc<sup>fl</sup>)/J) mice (11) were obtained from Andrew P. McMahon (Harvard University, Cambridge, MA). Generation and characterization of Nmyc<sup>E1F1</sup> as well as Math1-cre transgenic animals have been previously described (12, 13). Genotyping was done by PCR analysis using genomic DNA from mouse tail biopsies. Primers have been previously published. Mice were i.p. injected with 3 μg/g Dex (Sigma) daily; controls received equivalent volumes of saline vehicle.

Histology, in situ hybridization, and immunohistochemistry

For conventional histology and immunohistochemistry, mouse brains were formalin fixed, paraffin embedded, and
cut using standard protocols. For *in situ* hybridization, brains were perfused and post-fixed overnight with 4% paraformaldehyde/PBS. Tissue was then equilibrated in 0.5 mol/L sucrose/PBS (pH 7.4), embedded in optimum cutting temperature, and cut. *In situ* hybridization on frozen sections was done as previously published (13). For immunohistochemistry, sections were subjected to heat antigen retrieval at 99°C in 10 mmol/L sodium citrate buffer for all antibodies. Staining was done using the horseradish peroxidase (HRP)/3,3′-diaminobenzidine-based Envision+ staining system (DAKO) according to the manufacturer’s specifications. Immunofluorescence stainings were done using standard conditions with Alexa goat anti-rabbit and Alexa goat anti-mouse secondary antibodies (Molecular Probes). 4′,6-Diamidino-2-phenylindole was used to visualize cell nuclei. Primary antibodies were NeuN (Chemicon), phosphorylated histone H3 (pH3; Cell

Figure 1. Effects of dexamethasone treatment in Math1-cre:SmoM2 mice. A, medulloblastoma in 21-d-old Math1-cre:SmoM2 mice that were treated with 3 μg/g Dex (n = 4) from day 10 on had similar morphology than controls (n = 5), but were significantly smaller in size (P < 0.001). B, analysis of pH3, PCNA, and Caspase-3 revealed significantly less mitotic activity but increased apoptosis in Dex-treated animals. C, survival of treated animals was significantly enhanced as compared with controls (P < 0.02). D, RT-PCR analysis from tumor tissue showing similar levels of Nmyc, Gli1, and β-actin mRNA. Western blotting showed posttranslational changes in treated animals with unchanged levels of Gli1 and reduced levels of Nmyc proteins. CB, cerebellum; MB, medulloblastoma. Bar, 1 mm (A) and 100 μm (A, insets, and B).
Signaling), proliferating cell nuclear antigen (PCNA; Chemicon), and caspase-3 (Cell Signaling).

**Cell culture**

Primary cultures from Math1-cre:SmoM2 tumors were established by triturating tumor tissue from 21-day-old medulloblastoma-bearing mice in DMEM and plating the cells on poly-D-lysine–coated dishes. Cells were maintained in DMEM containing 10% FCS (Sigma) and treated with 200 μmol/L Dex and 10 μmol/L lactacystin (Calbiochem).

**Reverse transcription-PCR**

For RNA extraction, primary cultured tumor cells were homogenized in TRIzol (Invitrogen) before chloroform was added. After a 3-minute incubation, homogenates were centrifuged at 12,000 × g for 15 minutes. The aqueous phase was collected and precipitated with 350 μL of isopropanol. The precipitate was collected by centrifugation at 12,000 × g for 15 minutes and resuspended in 100 μL of RNase-free H2O. Before continuing with reverse transcription-PCR (RT-PCR), RNA was cleaned up with an RNeasy Kit (Qiagen). RT-PCR was done on isolated RNAs with an Advantage RT-for-PCR Kit (Clontech) and the following primers: Nmyc forward, 5'-ACAGCGGGGCGAAGTCG-3'; Gli1 reverse, 5'-CCTCAGCCCCAGTATCCCCAGTCG-3'. For β-actin, the Mouse β-Actin Control Amplimer Set (Clontech) was used.

**Western blotting**

To detect proteins by immunoblotting, nondenaturing lysates were prepared from CGNPs as described previously (5). Immunoblots were incubated overnight at 4°C with the following primary antibodies: cyclin D1 (Neomarkers, Ab-3), β-tubulin (Sigma-Aldrich), Gli1 (Santa Cruz), Nmyc (Calbiochem), PT58Nmyc (Abcam), PS54Nmyc (Abcam), GSK3B (BD Biosciences), PSer9GSK3α/β (Cell Signaling), and PT216GSK3β (Invitrogen). Subsequently, immunoblots were developed using HRP-conjugated antirabbit (Pierce) or antimouse (Jackson ImmunoResearch) secondary antibodies and enhanced chemiluminescence reagents (Amersham).

**Results and Discussion**

Systemic treatment of wild-type mice with Dex inhibits Shh-induced proliferation of CGNPs (9). To investigate whether Dex treatment would have an effect on medulloblastoma, we used an established Math1-cre:SmoM2 mouse model (2). These mice express a mutated Smoothened protein (SmoM2) in Math1-positive cerebellar granule cells and...
develop medulloblastomas with 100% penetrance and an average survival of 35 days.

We treated Math1-cre:SmoM2 animals with a daily i.p. dose of 3 μg/g Dex. Control animals received saline injections. Treatment was started at P10, a time point by which all CGNP have undergone neoplastic transformation in Math1-cre:SmoM2 animals (2). Macroscopic examination of the brains at day 21 revealed that Dex-treated animals had significantly smaller brains (data not shown), in keeping with previous studies (9). Whereas the tumor cell morphology of Dex-treated tumors and that of tumors from mice that received control injections for 11 days were similar, tumor sizes grossly differed between the two groups (Fig. 1A). Indeed, as measured by the weight ratio of the cerebellum including medulloblastoma and the total brain, medulloblastomas from Dex-treated animals (n = 4) were significantly smaller than those from controls (n = 5, P < 0.001). These data indicate that Dex treatment was able to inhibit the growth of Shh-associated medulloblastoma, and that such inhibition was disproportionate compared with the general growth of the brain. Analysis of pH3, PCNA, and cleaved caspase-3 expression revealed that mitotic activity was significantly reduced whereas apoptotic activity was significantly enhanced in Dex-treated tumors (P < 0.05, P < 0.02, and P < 0.05, respectively; Fig. 1B).

Next, we analyzed whether Dex treatment was associated with an altered survival of Math1-cre:SmoM2 animals and found that Dex-treated mice (n = 16) survived significantly longer than saline-treated mice (n = 15, P < 0.02; Fig. 1C). In particular, Dex-treated animals showed an average survival of 25 days after the start of treatment, whereas saline-treated mice already died after an average of 16 days.

To further decipher the mechanisms of Dex-induced inhibition of tumor growth, we asked whether the transcription of Shh-target genes in CGNP was affected. Semiquantitative RT-PCR analysis did not reveal any differences in mRNA levels of Nmyc or Gli1 (Fig. 1D). In contrast, Nmyc protein levels were reduced in tumors from Dex-treated animals by almost 30%, whereas Gli protein levels were unchanged. These results indicate that treatment of medulloblastoma with Dex does not regulate the transcription of primary target genes, Gli and Nmyc. It has, however, a significant effect on Nmyc protein levels. Although these findings are in agreement with previous findings in developing CGNP (9), we note that the dose of Dex required to achieve these effects in transformed CGNP of medulloblastoma is 30-fold higher. This difference presumably reflects the strong activation effects of SmoM2.

Previous data have shown that Nmyc and Gli proteins play critical, and possibly overlapping, roles in driving the proliferation of cerebellar granule cell precursors (4, 5). To assess a specific requirement for Nmyc in CGNP per se, we generated Math1-cre:NmycF/F::SmoM2 mice and assessed their survival versus Math1-cre:SmoM2 tumor-prone animals. As shown in Fig. 2A, 15% of the Math1-cre:NmycF/F::SmoM2 mice displayed cerebella with a dramatically altered morphology and a nearly complete depletion of granule neurons. Most importantly, they did not develop medulloblastoma, indicating that SmoM2-induced medulloblastomas require Nmyc function in CGNPs. Interestingly, in the 85% of
Math1-cre:Nmyc<sup>FL/FL</sup>SmoM2 mice that died from medulloblastoma (Fig. 2B), we observed expression of full-length Nmyc (Fig. 2C). To see whether this was caused by an incomplete genomic recombination of the Nmyc allele, we analyzed genomic DNA from tumors of different genotypes using primers picking up wild-type sequences, floxed sequences, and deleted Nmyc sequences. As indicated in Fig. 2C, a substantial part of the Math1-cre:Nmyc<sup>FL/FL</sup>SmoM2 tumors retained the floxed allele due to insufficient recombination, a phenomenon that is well known for the conditional knockout of genes that are required for tumor growth (14). Our results are in agreement with previously described interactions of Shh and Myc family members (15) as well as with Nmyc requirements during cerebellar development (12) and in medulloblastoma (16), but make two novel points: First, we used Math1 promoter sequences, which are more specific for granule cell precursors than the Nestin (which is expressed in multipotent precursors; ref. 17) or NeuroD2 sequences (which is expressed in CGNP and molecular layer interneurons; ref. 18). Second, because our system combines Nmyc deletion and SmoM2 activation simultaneously, our data indicate a cell-autonomous requirement for Nmyc in CGNPs.

The results above suggested the possibility that the reduced levels of Nmyc proteins in medulloblastoma after Dex treatment (Fig. 1D) could account for reduced tumor growth and enhanced survival of Math1-cre:SmoM2. During normal CGNP development, Nmyc protein destabilization is mediated by a cyclin-dependent kinase 1/cyclin B complex that phosphorylates Nmyc at S54 followed by terminal phosphorylation of T58, which promoted proteasome-mediated degradation. Because GSK3β phosphorylates Nmyc at T58, we assessed GSK3β activation in Dex-treated medulloblastoma cells. As shown in Fig. 3B, levels of P<sub>S54</sub>αβGSK3β, but not P<sub>T58</sub>αβGSK3β, were decreased, indicating an increased activity of GSK3β. We conclude that Dex may activate GSK3β, which, in turn, promotes the phosphorylation of Nmyc at T58 and the degradation of Nmyc proteins. Together, these findings suggest that glucocorticoid signaling inhibits Shh signaling in medulloblastoma by promoting degradation of Nmyc.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

FoxM1 regulates mitotic entry and prevents spindle defects in cerebellar granule neuron precursors. Mol Cell Biol 2007;27:8259–70.


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