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Tumor necrosis-initiated complement activation stimulates proliferation of medulloblastoma cells

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Abstract

Objective and design—We sought to determine the effect of necrosis-induced activation of the complement protein C3 in medulloblastoma.

Materials/methods—Twelve medulloblastoma surgical specimens were evaluated for complement activation using immunohistochemistry, with H&E stains performed on adjacent tissue sections to determine the relationship of complement activation to necrotic tissue. Flow cytometry and Western blot were performed on three established medulloblastoma lines and one surgically-procured cell culture to determine expression of C3a receptor (C3aR) in medulloblastoma. In vitro proliferation of siRNA C3aR knockdown cells was compared to that of control siRNA cells with cell line Daoy.

Results—Three surgical specimens were found to have necrosis on H&E sections. In each case, iC3b staining was identified on adjacent sections, limited to the necrotic region. In no case did necrosis occur without iC3b staining on adjacent sections. C3aR protein was demonstrated on both the three established cell lines and on the surgical culture. Proliferation assays of Daoy cells with siRNA knockdown vs. control siRNA revealed significantly reduced proliferation at 72 h (p = 0.001).

Conclusions—Necrosis is associated with complement activation in medulloblastoma. Medulloblastoma cells express C3aR, and siRNA-mediated knockdown of C3aR inhibits proliferation of these cells in vitro.

Keywords

Medulloblastoma; Complement; Necrosis; Proliferation

Introduction

The complement system is composed of a collection of proteins ubiquitous to plasma and interstitial fluid in humans and other organisms, and plays an important role in the immune response to pathogens and inflammation [1–4]. The cascade of downstream events is initiated by three separate pathways—the classical, alternative, and mannose-binding lectin pathways—which converge at formation of C3 convertase, which enzymatically cleaves C3 protein into effector molecules C3a and C3b (Fig. 1). Necrosis has been demonstrated as a potent initiator of the complement cascade (primarily through the classical and alternative pathways), and may be the catalyst for initial complement deposition in diseased tissues [3, 5–8]. Although investigation of complement's role in disease has been mostly focused on derangements of previously-characterized immune functions of the system, recent studies have demonstrated a relationship between complement proteins and the regulation of cell growth, turnover, and tissue repair and regeneration [3, 4, 9–12]. Specifically, the anaphylotoxin effector proteins C3a and C5a have recently been implicated in the stimulation of progenitor cells to repair and regenerate tissues in several organ systems in the body, including the central nervous system [1, 2, 13–20]. In addition, several authors have

also investigated the role of complement in the development and maintenance of neoplasms throughout the human body, including the central nervous system [21–24]. Thus, beyond formation of membrane attack complexes on cells destined for destruction, the role of complement includes assistance with proliferation of progenitor as well as neoplastic cells in order to repair damage from inflammation, infection, and cellular death.

Medulloblastoma is the most common malignant brain tumor in children. It is thought to develop from neuronal progenitor cells of the cerebellum [25, 26]. These tumors often contain necrotic areas, the presence of which on histologic examination has grading significance in the current WHO scheme. Several studies have indicated that the presence of necrosis independently predicts a poorer prognosis in these patients [27–31].

While this correlation has been attributed to more aggressive tumors outgrowing their vascular supply, our study presents evidence that necrosis may not be a biologically inert epiphenomenon, and may in fact play a role in driving the growth of these tumors. In this paper, we propose that necrotic medulloblastoma tissue activates the complement cascade, releasing effector molecules (including C3a) as split products, which interact with C3a receptors present on tumor cells and consequently promote the proliferation of these cells.

Materials and methods

Cell Lines

D283 Med (HTB-185™), Daoy (HTB-186™), and D341 Med (HTB-187™) cell lines were purchased from American Type Culture Collection (ATCC®). The surgical specimen was disaggregated with mincing and a single-cell suspension was obtained by passing the sample through a 70-μm cell strainer. All cell lines were established in culture prior to experimentation.

Cell culture

Cell lines were maintained at 37 °C, 95 % room air, and 5 % CO_2, in DMEM supplemented with fetal bovine serum (FBS) (10 % for HTB-185 and 186; 20 % for HTB-187) and 5 % penicillin–streptomycin (100 units/mL penicillin, 100 μg/mL streptomycin). Cell lines were cultured, grown to approximately 80 % confluence, washed in 1 % D-PBS (pH 7.4), and then trypsinized. Cells were subsequently centrifuged, resuspended in serum-free and antibiotic-free DMEM, and counted using a hemacytometer using the Trypan blue methodology.

Immunohistochemistry

Immunohistochemistry was performed on tumor specimens obtained with informed consent from the brain tumor bank with Institutional Review Board approval. The tissue was paraffin-embedded shortly after operative resection, sectioned via microtome, and mounted. Prior to antigen retrieval, sections were deparaffinized in a rice steamer using deionized (DI) water for 20 min. The sections then underwent background block for 10 min (Cell Marque®), a DI water washing, and peroxide block for 10 min (Cell Marque®), prior to incubation overnight at 4 °C with either the iC3b neoantigen monoclonal antibody (Quidel®)

at 1:4,000 (0.25 μg/mL) or mouse IgG control (Sigma®) at 1:800 (0.25 μg/mL). After washing with PBS, the sections were incubated with HiDef amplifier (Cell Marque®) for 10 min, washed again with PBS, and incubated for another 10 min with HiDef HRB polymer (Cell Marque®). After another PBS wash, the sections were developed with DAB Chromogen (Biocare®), washed with DI water, and finally were counterstained with immunohematoxylin (American Master Tech®).

Flow cytometry

Cells were established in a single cell suspension in PBS supplemented with 2 % BSA. Cells were incubated with PE-conjugated anti-Human C3a Receptor and corresponding isotype control (BD Pharmingen®) for 45 min at room temperature protected from light. The cells were then washed with PBS. Immediately after washing, flow cytometry was performed with the BD FACSCalibur™ system (BD Biosciences®). Data was analyzed using BD CellQuest™ Pro.

Western blotting

Cells were lysed in cell lysis buffer (Cell Signaling Technology®), and lysates were cleared of insoluble material using centrifugation, then boiled in SDS sample buffer. Equal amounts of protein were loaded and electrophoresis was performed through SDS-PAGE gels (Bio-Rad©). Resolved proteins were transferred to PVDF membranes (Millipore©), which were blocked with 5 % BSA dissolved in TBS containing 0.02 % Tween 20, then incubated with primary antibody C3aR (H300) (Santa Cruz©) diluted in the same buffer. Membranes were washed and subsequently incubated with anti-rabbit-HRP antibody (Cell Signaling Technology®) and β-actin-HRP mouse monoclonal antibody (Sigma-Aldrich®). The blots were developed with Super Signal West Pico Chemiluminescence reagent (Pierce®). HeLa Whole Cell Lysate (sc-2,200, Santa Cruz©) was used as a positive control.

Cell transfection

Human C3aR siRNA and universal scrambled siRNA were purchased from OriGene Technologies, Inc. Lipofectamine RNAIMAX transfection reagent and OPTI MEM media were purchased from Life Technologies™. The three human medulloblastoma cell lines detailed previously were grown in culture per vendor recommendations. Using cells at passage three or four, transfection was accomplished using the C3aR siRNA and the Lipofectamine RNAIMAX system mentioned above and optimized per manufacturer's instructions.

Cell proliferation assay

Cell proliferation rate experiments were performed by plating siRNA-transfected cells (as described above) in a 96-well plate and exposing them to experimental conditions for 0–72 h. At 24, 48, and 72 h, proliferative rates in each experiment were determined using the XTT assay as mentioned above (detailed description available from www.ATCC.org). Essentially, the cells were incubated with a second-generation tetrazolium dye. The dye is reduced at the cell surface, resulting in the formation of a soluble formazin product with an absorption maximum at 475 nm. Corrections were made for both blank and non-specific background

absorption. The resulting corrected absorbance units were plotted vs. time. Increased proliferation was indicated by higher absorbance values and decreased proliferation by lower values. Multiple runs were used to create groups of size $n = 5$.

Statistical analysis

Time points in the proliferation assay were compared using Student's t test, with $n = 5$ for each time point. Comparisons were made between control siRNA and C3aR siRNA samples. Western blots were analyzed using Image Studio 3.1 (Li-Cor, Inc®). Student's t test was used to compare results of five western blots for C3aR; comparisons were made between control siRNA and C3aR siRNA lanes. In all statistical analyses, results were considered significant if $p < 0.05$.

Results

Necrosis activates the complement system in medulloblastoma tissue

Since necrosis is a well-known initiator of complement system activation, we hypothesized that products of complement activation would be evident in medulloblastoma tissue, localized mostly to necrotic regions. We examined 12 paraffin-fixed and sectioned medulloblastoma specimens in series, alternating staining on adjacent sections with H&E and immunohistochemistry using an antibody against the inactivated complement split product C3b (denoted iC3b). Necrosis was present in 3 out of 12 specimens. In these specimens, the highly eosinophilic regions on H&E stained sections (demonstrating necrosis) correlated with iC3b-staining regions on adjacent sections (Fig. 2). IHC staining for iC3b was notably absent in areas of viable-appearing tumor cells on adjacent H&E-stained sections, suggesting that C3 is activated by necrotic tissue but not by medulloblastoma cells in general.

Medulloblastoma cells express the C3aR complement receptor

To document the expression of C3aR in medulloblastoma cells, we performed flow cytometry on three established medulloblastoma cell lines (Daoy, D283, and D341) and one cultured surgical case (Fig. 3). We also performed Western blot on the three established medulloblastoma cell line lysates (Fig. 4). In both experiments, C3aR was identified on medulloblastoma cells in all instances, suggesting that C3aR expression is a common phenomenon in medulloblastoma cells.

C3aR blockade decreases medulloblastoma cell proliferation

We then performed transfection experiments to determine the effect of C3a receptor activation on medulloblastoma cells. Fetal bovine serum is a source of complement proteins including C3, and we anticipated that the in vitro cell population would have complement activation through baseline cell death in culture. Consequently, we hypothesized that reduced C3a receptor levels would affect proliferative rate.

At 72 h, we found statistically significant reduction of proliferation in C3aR siRNA-treated cells as compared to control siRNA-treated cells in cell line Daoy ($p = 0.001$; Fig. 5). We performed Western blots to determine whether C3aR expression in the experimental group

had in fact been reduced, which confirmed modest knockdown [45.0 % mean reduction in expression via densitometry (range 30–53 %)]. That is, cells with reduced C3aR experienced reduced proliferation, suggesting that C3a receptor activation leads to proliferation of medulloblastoma cells. We further conducted linear regression assessing for a correlation between reduction in proliferation and percent knockdown; there was a weak correlation ($r = -0.188$), suggesting that increasing knockdown decreased proliferation, but this was not statistically significant ($p = 0.76$). This may be explained by the sample number and the limited variance in knockdown across experiments.

Discussion

Recent research has challenged the idea that the major function of the complement system is to destroy pathogens and mediate inflammation. Several authors have expanded on the concept that this system is a bridge between the innate and adaptive immune functions, presenting compelling evidence that the complement system is an essential component in the repair and regeneration of injured tissues [3, 4, 6, 11, 14, 18, 22, 32–34]. In addition, recent research has suggested roles of the complement system in aiding tumor cell proliferation through immunosuppression, mitogenic pathways, anti-apoptosis signaling, angiogenesis, and other tumorigenic processes [10–12, 21, 35, 36].

In this study, we provide data suggesting that necrosis within medulloblastoma is associated with cleavage of C3 to C3a and C3b, and that C3aR is expressed in both medulloblastoma tissues and in established cell lines. Finally, we note that reduced expression of C3aR leads to reduced proliferation in vitro, with cell culture media as a source of complement proteins (including C3) and cell death in culture as an instigator of complement activation (forming C3a).

The fact that the C3a receptor is expressed on medulloblastoma cells is not altogether surprising, given the presence of this receptor on a number of central nervous system cells, including astrocytes and neurons [37], in locations throughout the brain, including the cerebellum [38]. Further, the association of complement activation with necrosis has been previously documented [4]. Expanding upon this, however, our findings suggest that medulloblastoma cells possess the molecular components necessary to respond to necrosis-associated C3a receptor activation, and that they do so by proliferating. Thus, it may be the case that these tumor cells respond to tissue injury just as normal tissue would respond to such an insult. Our study did not attempt to elucidate the mechanism by which C3a receptor activation leads to cell proliferation, though this is in an area of future inquiry.

Many of the present treatments for medulloblastoma are cytolytic in nature. The fact that necrosis may potentially be a source of pro-proliferative stimuli provides one possible insight into why these therapies fail in some patients. The obvious challenge in translating these findings to the clinic involves the fact that necrosis is an ongoing biologic event in these tumors, and thus this target might require ongoing blockade for an extended period of time, necessitating long-term treatment.

Conclusions

We provide evidence that necrosis within medulloblastoma may have deleterious effects through activation of the complement protein C3. When C3a receptor expression is reduced, cellular proliferation is markedly decreased in vitro. The complement system has been linked to other cancers as well, which prompts the question of whether oncotherapies that cause necrosis in general may have a mixed effect in tumor control. Further studies are warranted, but the development of therapy targeting C3aR may hold significant therapeutic promise in medulloblastoma and could even be relevant in the treatment of other cancers of the CNS and throughout the body. These are interesting topics for future study.

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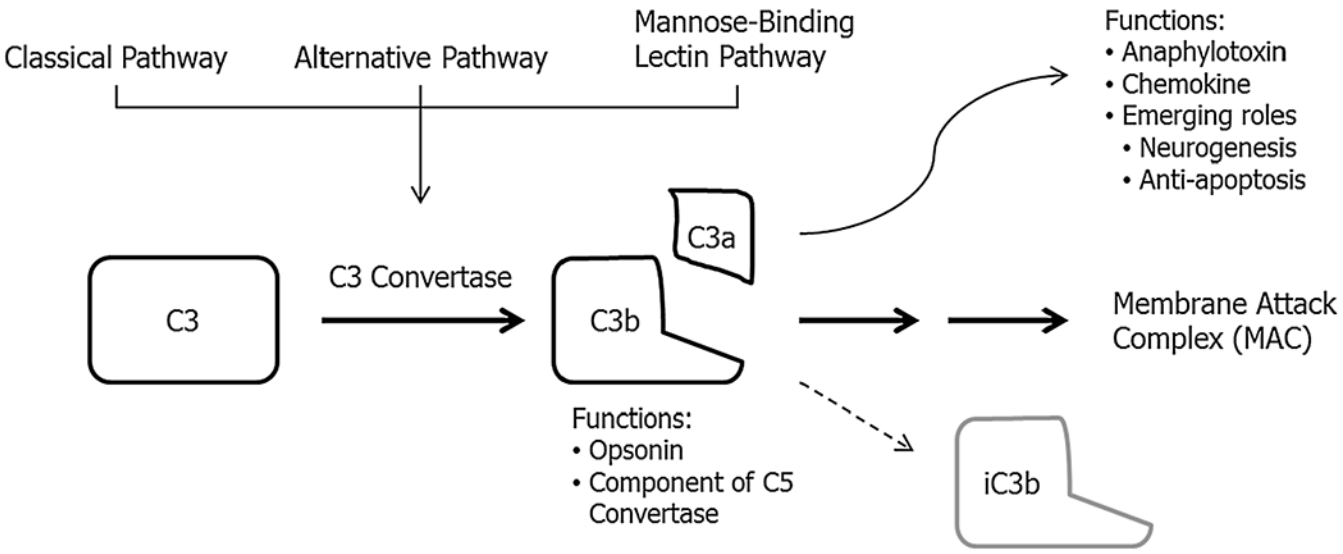

Fig. 1.

The complement cascade is comprised of three pathways which converge at formation of the C3 convertase enzyme. C3 convertase cleaves the complement C3 protein into C3a and C3b, each of which has several important functions in the immune response. C3a has both paracrine and endocrine effects. C3b has local effects and is subsequently inactivated to insoluble molecule iC3b

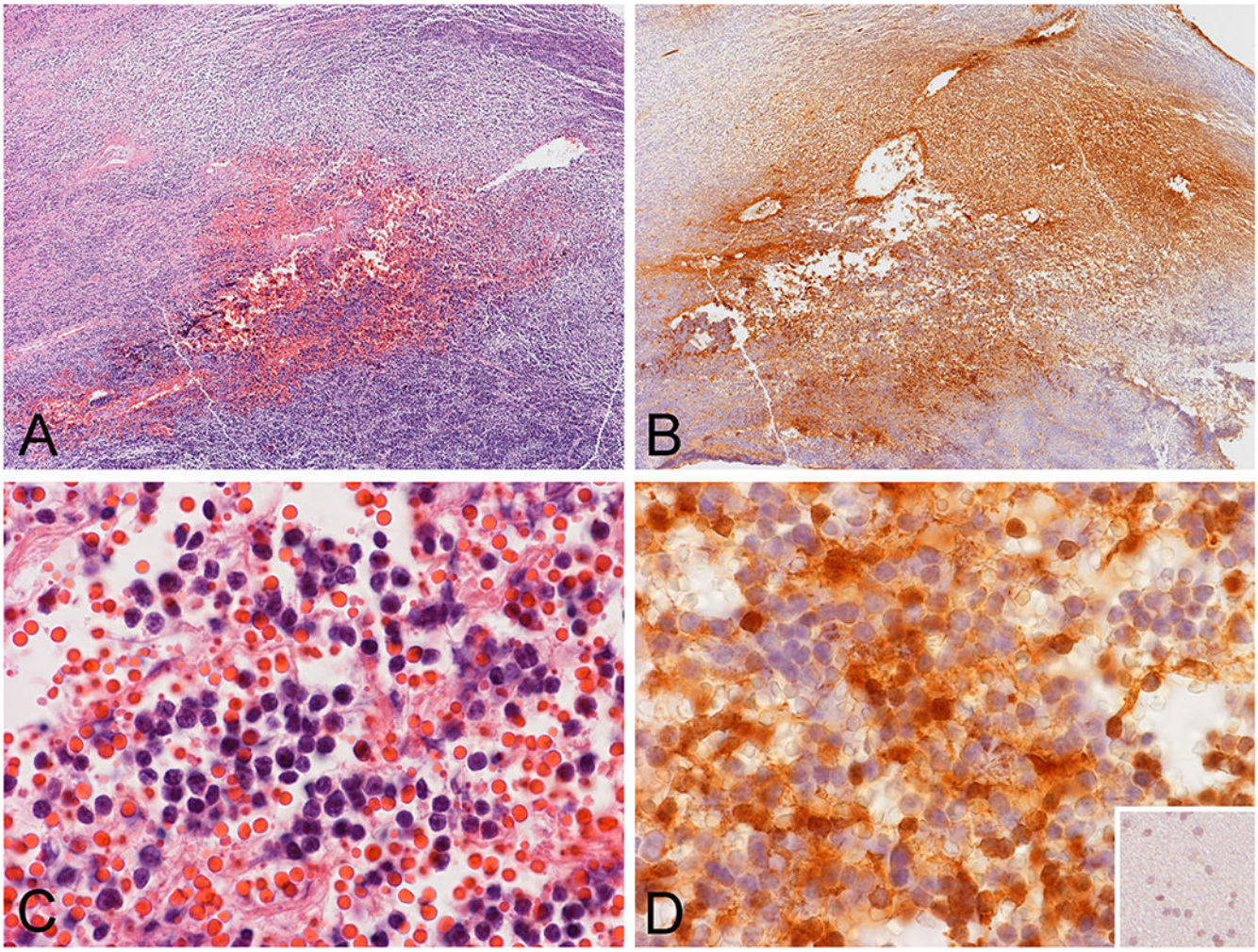

Fig. 2.

Representative case demonstrating evidence of complement activation within area of necrosis. **a** H&E of a medulloblastoma surgical specimen at low power (×4) demonstrates focal necrosis with hemorrhage. **b** iC3b IHC shows positivity in the same region on adjacent section. **c** At high power (×60), H&E reveals viable tumor cells interspersed with erythrocytes and necrosis. **d** iC3b IHC at high power shows staining of necrosis but not viable tumor cells. In addition, expected staining of erythrocytes is seen. A section obtained from a case of temporal epilepsy (inset) demonstrates absent staining for iC3b in the setting of gliosis

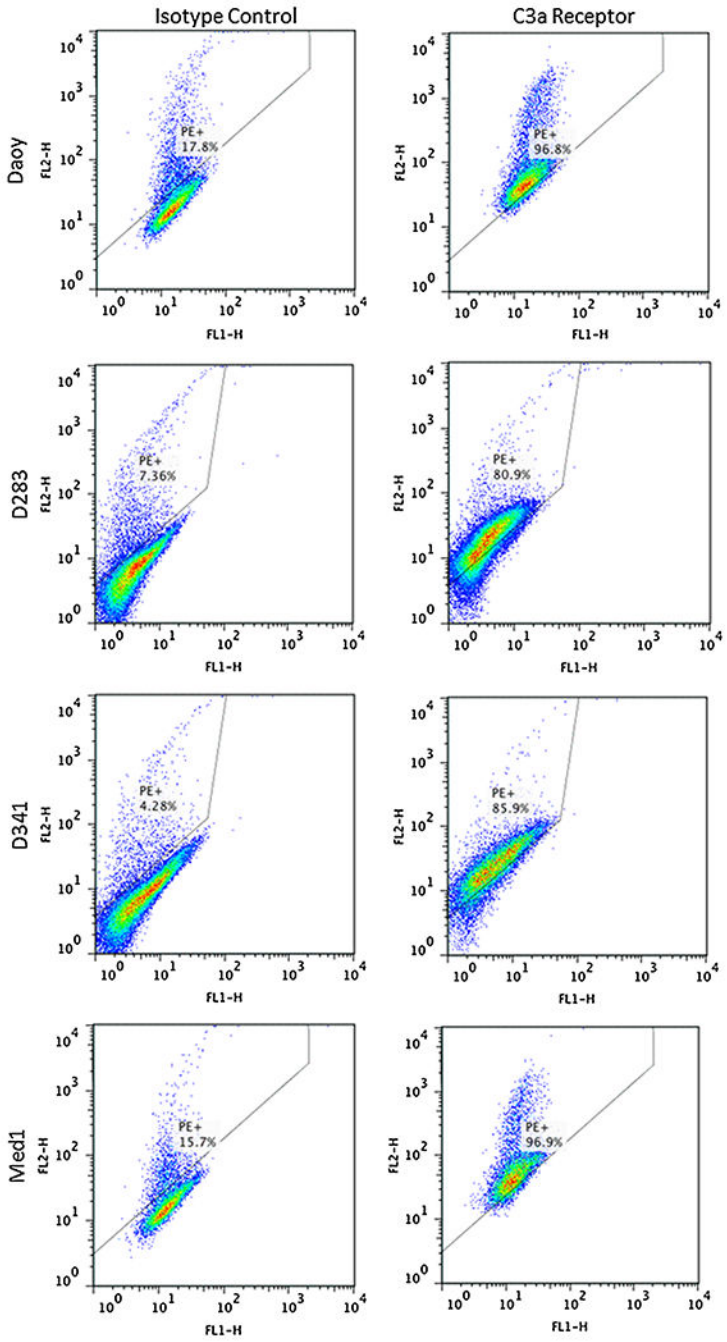

Fig. 3.

Presence of C3aR measured by flow cytometry in four cell lines. With cells gated according to the isotype control (*left column*), the majority (>80 %) of cells are positive for C3aR in all cases (*right column*)

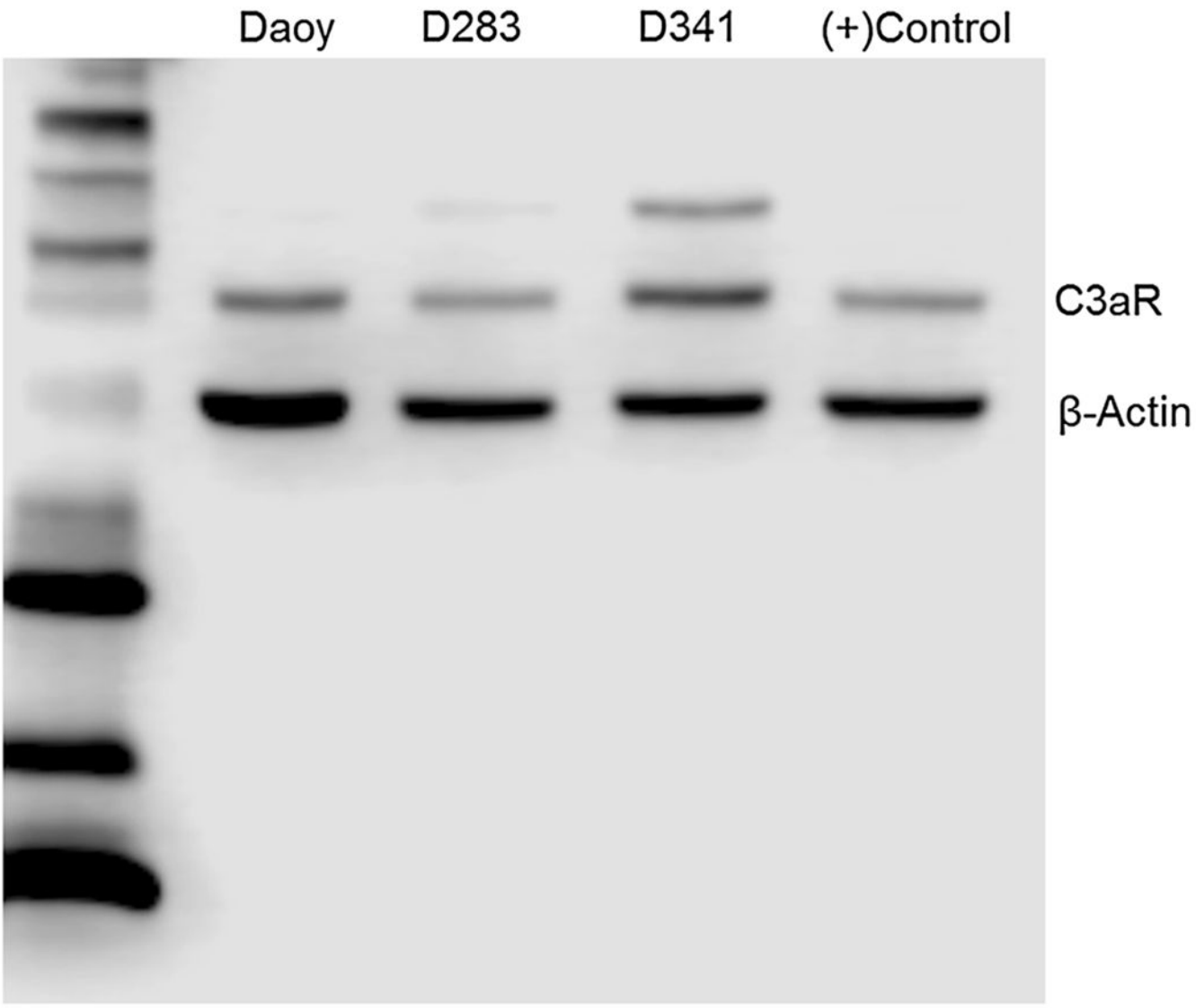

Fig. 4.

Western blot in D283, D341, and Daoy medulloblastoma lines demonstrates expression of C3a receptor (C3aR). HeLa whole cell lysate (*right lane*) was used as a positive control per manufacturer's specifications

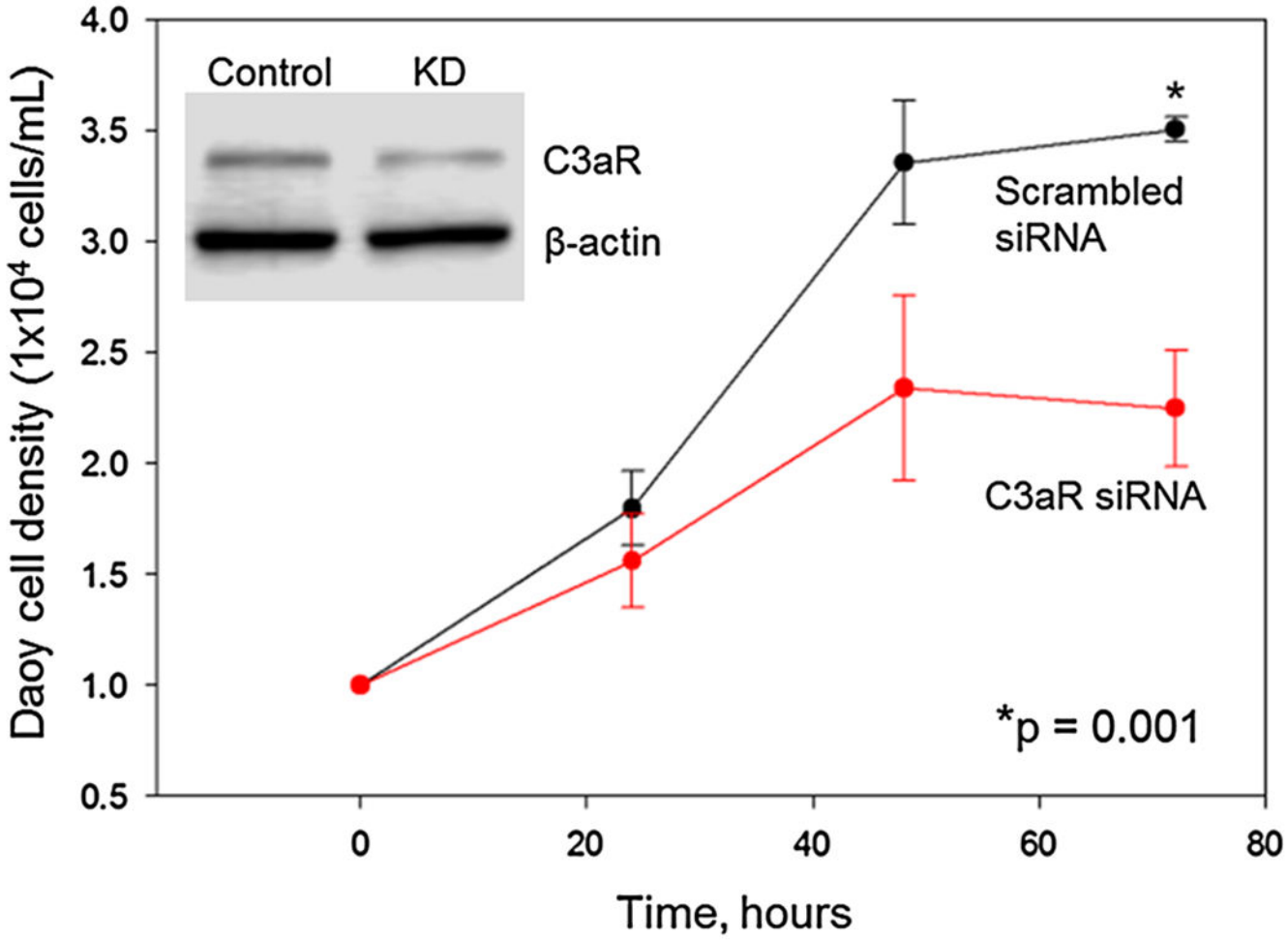

Fig. 5.

Aggregated siRNA-mediated knockdown assay against C3aR in Daoy cells. The C3aR siRNA-treated cell population shows statistically significant reduction in proliferation of C3aR-knockdown cells ($p = 0.001$ at 72 h). Western blot confirms modest knockdown