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Inhibition of Microsomal Prostaglandin E Synthase-1 in Cancer-Associated Fibroblasts Suppresses Neuroblastoma Tumor Growth

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ABSTRACT

Despite recent progress in diagnosis and treatment, survival for children with high-risk metastatic neuroblastoma is still poor. Prostaglandin E_2 (PGE_2)-driven inflammation promotes tumor growth, immune suppression, angiogenesis and resistance to established cancer therapies. In neuroblastoma, cancer-associated fibroblasts (CAFs) residing in the tumor microenvironment are the primary source of PGE_2. However, clinical targeting of PGE_2 with current non-steroidal anti-inflammatory drugs or cyclooxygenase inhibitors has been limited due to risk of adverse side effects. By specifically targeting microsomal prostaglandin E synthase-1 (mPGES-1) activity with a small molecule inhibitor we could block CAF-derived PGE_2 production leading to reduced tumor growth, impaired angiogenesis, inhibited CAF migration and infiltration, reduced tumor cell proliferation and a favorable shift in the M1/M2 macrophage ratio. In this study, we provide proof-of-principle of the benefits of targeting mPGES-1 in neuroblastoma, applicable to a wide variety of tumors. This non-toxic single drug treatment targeting infiltrating stromal cells opens up for combination treatment options with established cancer therapies.

1. Introduction

Cancer-related inflammation is a complex tumor-promoting interaction between cancer cells and stromal cells of the microenvironment. These interactions contribute to tumor progression by inducing angiogenesis, providing the tumor with growth factors, and by impairing anti-tumor immunity by immunosuppressive mechanisms [1–3]. Neuroblastomas are neural crest derived embryonic tumors of the sympathetic nervous system that are amongst the most common and deadliest tumors in young children. Children with high-risk neuroblastoma still have a poor prognosis and despite intensified multi-modal therapy only 40% -50% of these children survive their disease [4,5]. High-risk neuroblastomas include tumors harboring an amplification of the *neuroblastoma MYC* (*MYCN*) oncogene often present in younger children, and another subset of tumors with a deletion of the long arm of chromosome 11 (11q-deletion) usually presenting in children older at diagnosis [6]. An immunosuppressive microenvironment has been described in neuroblastoma and recent studies have shown that the high-risk tumors possess a microenvironment with pro-inflammatory features [5,7–10].

Prostaglandin E_2 (PGE_2) is a bioactive lipid mediator. Dysregulation of PGE_2 biosynthesis has been found in a variety of malignancies. Through its pro-inflammatory actions PGE_2 contributes to a tumor-promoting microenvironment by inducing growth factors and angiogenic factors that stimulate tumor growth. At the same time PGE_2 contributes to an immunosuppressive milieu leading to inhibition of anti-tumor immunity [11–13]. In addition, PGE_2 contributes to resistance to radiation and chemotherapy where PGE_2 released from dying cells sustains tumor repopulation [14,15]. PGE_2 is formed by the conversion of arachidonic acid by the cyclooxygenases (COX-1 and COX-2) into prostaglandin H_2 that is further converted to PGE_2 by the terminal synthase, microsomal prostaglandin E synthase-1 (mPGES-1). The biological effect of PGE_2 is conducted *via* four G-protein coupled receptors EP1-EP4. Clinical use of COX inhibitors in oncology, *i.e.* non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors (Coxibs), which reduce the production of PGE_2 and other prostanoids important for normal cellular functions, has been hampered by severe side effects on the gastrointestinal and cardiovascular systems. This has led to the development of mPGES-1 inhibitors that selectively target PGE_2 production as a strategy to achieve the anti-tumorigenic properties of PGE_2-blockade [16–21]. Due to phenotypic differences between murine and human mPGES-1, inhibitors developed towards the human enzyme are ineffective

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towards the murine enzyme hindering pre-clinical studies in mouse models [22]. However, recently dual murine and human mPGES-1 inhibitors have been characterized in mouse models of inflammation [23,24].

We have reported that high-risk neuroblastomas present an immunosuppressive microenvironment and that infiltrating cancer-associated fibroblasts (CAFs) are responsible for the mPGES-1 expression in these tumors [25]. Drugs that specifically target non-neoplastic cells within the microenvironment have been suggested as a treatment option to further improve survival and quality of life [5,9,26]. In the present study, we investigated the significance of PGE_2 producing CAFs and the specific effects of pharmacological mPGES-1 inhibition in the neuroblastoma microenvironment.

2. Materials and Methods

2.1. Patient Material

Neuroblastoma tumor tissues were obtained at Astrid Lindgren Children's Hospital, Karolinska University Hospital, Sweden, and were immediately (<60 min) fresh frozen at surgery after a minimal of two weeks after any treatment. Relevant informed consent was obtained according to the ethical approval from the Karolinska University Hospital Research Ethics Committee (approval no 2009/1369–31/1 and 03/736).

2.2. Chemicals

The mPGES-1 inhibitor Compound III (CIII) [23] was synthesized by NovaSAID AB. CIII was resuspended (1% Tween80, 0.5% carboxymethyl cellulose, 0.9% NaCl) at a final concentration of 4 mg/ml before use.

2.3. Cell Lines

The human neuroblastoma cell lines SK-N-AS (ATCC Cat# CRL-2137, RRID:CVCL_1700) were cultured as previously described [27] and authenticated using short tandem repeat analysis. The normal human dermal fibroblast cell line (NHDF, Cat# C-12300, PromoCell) was grown in RPMI supplemented with 10% FBS, L-glutamate and antibiotics. All cell lines were tested for Mycoplasma using PCR analysis.

2.4. Immunohistochemical Analysis of Neuroblastoma Tumor Tissue

Frozen tumors were sectioned in 7 μm thin sections using a cryostat and fixed in 2% formaldehyde for 20 min. Dilutions and washes were performed using PBS containing 0.1% saponin, pH 7.4. Endogenous peroxidase activity was blocked using 1% H_2O_2 and biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories). Tumor sections were incubated with primary antibody dilutions, containing 3% normal serum (human or mouse depending on tissue origin), overnight in room temperature (RT). Primary antibody concentrations are listed in supplementary Table S1. Following incubation with 1% goat serum (or horse serum depending on secondary antibody) for 15 min, sections were incubated with biotin-conjugated secondary antibody, containing 1% goat or horse serum and 3% normal serum, for 30 min in RT (goat α-rabbit IgG, 1:1600, Vector Laboratories Cat# BA-1000, RRID:AB_2313606; horse α-goat IgG, 1:300, Vector Laboratories Cat# BA-9500, RRID:AB_2336123; goat α-rat IgG, 1:200, Vector Laboratories Cat# BA-9400, RRID:AB_2336202). After incubation with ABC complex (Elite ABC kit, Vector Laboratories) the sections were developed for 6 min using Diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories) as chromogen. Sections were counterstained with Mayer's Hematoxylin (Histolab). At least three tumors from each treatment group were quantified for CD206, CD31, Ki-67 and PDGFRβ expression using Leica Qwin IM500 software as described previously (39). For immunofluorescent staining, sections were washed for 10 min and blocked in 20% normal serum for 45 min in RT. Sections were then incubated with primary antibody mixture over night in RT. Slides were washed and incubated with secondary antibodies conjugated with fluorescent dye in RT for 30 min (goat α-rabbit IgG (H+L), Alexa Fluor 488, 1:1000, Thermo Fisher Scientific Cat# A-11070, RRID: AB_2534114; goat α-mouse IgG (H+L), Alexa Fluor 594, 1:1000, Thermo Fisher Scientific Cat# A-11020, RRID:AB_2534087). Following additional washes, the sections were counterstained with Hoechst diluted 1:1000 for 30 s and washed in PBS. Sections were finally mounted in glycerol with PBS. Dilutions and washes were performed using PBS containing 0.1% saponin, pH 7.4.

2.5. Analysis of Prostanoids and CIII by Liquid Chromatography Tandem Mass Spectrometry

Prostanoids and CIII in plasma and tumors were extracted and analyzed to a large extent according to [28]. Working on ice, plasma (100 μl) and tumor tissue (200–400 mg) were spiked with 100 μl deuterated internal standards of 6-keto-$PGF_{1\alpha}$-d4, $PGF_{2\alpha}$-d4, PGE_2-d4, PGD_2-d4, TxB_2-d4, and 15-deoxy-Δ12,14PGJ_2-d4 (Cayman Chemical) in ethanol and made acidic with 500 μl 1% formic acid (FA) in methanol. The tumors were homogenized by mechanical force with a pellet pestle (Kontes) and liquid extraction was performed by addition of 400 μl 1% FA in methanol followed by centrifugation at 2500 g for 5 min (4 °C) and collection of supernatants. The liquid extraction was repeated once. The extracted supernatants were evaporated to dryness under vacuum and then reconstituted in 1 ml 0.05% FA, 10% methanol in MilliQ water. Solid-phase extraction (SPE) was performed by loading samples on Oasis HLB 1cm^3 30 mg cartridges (Waters) that had been preconditioned with 100% methanol and 0.05% FA in MilliQ. The SPE columns were washed once with 10% methanol in MilliQ followed by elution in 1 ml 100% methanol. The samples were evaporated to dryness under vacuum and stored at −20 °C until reconstituted in 30 μl 20% acetonitrile prior to analysis with liquid chromatography tandem mass spectrometry (LC-MS/MS). The injection volume was 10 μl and analytes of interest were quantified using a triple quadrupole mass spectrometer (Acquity TQ detector, Waters) equipped with an Acquity H-class UPLC (Waters). Separation was performed on a 50 × 2.1 mm Acquity UPLC BEH C18 column 1.7 μm (Waters) with a 12 min stepwise linear gradient (20–95%) at a flowrate of 0.6 ml/min with 0.05% FA in acetonitrile as mobile phase B and 0.05% FA in MilliQ as mobile phase A. Individual prostanoids were measured in negative mode [28] and CIII (*m/z* 384 > 341, Fig. S2) in positive mode with multiple reaction monitoring method. Data were analyzed using MassLynx software, version 4.1, with internal standard calibration and quantification to external standard curves.

2.6. Inhibition of mPGES-1 in Xenograft Tumor Bearing Mice

4–8 weeks old female NMRI nu/nu mice obtained from Taconic Laboratories, were maintained under pathogen free conditions and given sterile water and food *ad libitum*. The xenograft mice experiments were approved by the regional ethics committee for animal research (approval N231/14) in accordance with the Animal Protection Law (SJVFS 2012:26).

Each mouse was inoculated with 10^7 or 10^6 SK-N-AS cells in the right flank. When tumors from mice inoculated with 10^7 cells reached a volume of 150 mm^3 the animals were randomized by lottery assignment, either receiving 50 mg/kg CIII ($n = 6$), 100 mg/kg CIII ($n = 6$), Vehicle (0.5% Carboxymethyl cellulose, $n = 5$) or no treatment ($n = 5$). The drugs were administered intraperitoneal (i.p.) and the animals were treated for eight consecutive days. 30 mice were inoculated with 10^6 cells, 10 animals were randomized to receive 50 mg/kg from the day of injection, referred to as early treatment of CIII (CIII-ET). The remaining 20 mice were randomized by lottery assignment when tumors reached a volume of 200 mm^3 to either receive 50 mg/kg CIII ($n = 10$) or no treatment ($n = 10$). CIII was administered i.p. daily. All the

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