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Role of Cyclooxygenase-2 Pathway in Creating an Immunosuppressive Microenvironment and in Initiation and Progression of Wilms' Tumor

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Abstract

Wilms' tumors (WT), which accountfor 6% of all childhood cancers, arise from dysregulated differentiation of nephrogenic progenitor cells from embryonic kidneys. Though there is an improvement in the prognosis of WT, still 10% of patients with WT die due to recurrence. Thus more effective treatment approaches are necessary. We previously characterized the inflammatory microenvironment in human WT and observed the robust expression of COX-2. The aim of this study was to extend our studies to analyze the role of COX-2 pathway components in WT progression using a mouse model of WT. Herein, COX-2 pathway components such as COX-2, HIF1-α, p-ERK1/2, and p-STAT3 were upregulated in mouse and human tumor tissues. In our RPPA analysis, COX-2 was up-regulated in M15 cells after Wt1 gene was knocked down. Flow cytometry analysis showed the increased infiltration of immune suppressive inflammatory cells such as pDC's and Treg cells in tumors. The chemotactic chemokines responsible for the infiltration of these cells were also induced in CCR5 and CXCR4 dependent manner respectively. The immunosuppressive cytokines IL-10, TGF-β, and TNF-α were also up-regulated. Furthermore, more pronounced Th2 and Treg induced cytokine response was observed than Th1 response in tumors. Basing on all these evidences it is speculated that COX-2 pathway may be a beneficial target for the treatment of WT. It may be most effective as an adjuvant therapy together with other inhibitors. Thus, our current study provides a good rationale for initiating animal studies to confirm the efficacy of COX-2 inhibitors in decreasing tumor cell growth in vivo.

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Introduction

Wilms' tumor (WT), a pediatric tumor of the kidney, is the second most common cancer of children, accounting for approximately 6% of all childhood cancers [1]. While there has been slight improvement in the prognosis of WT in recent years, 10% of patients with WT still experience disease recurrence, and many of those die of their disease. Thus, the development of new, more effective approaches to treat this lethal malignancy is of considerable interest. Though mutations in WT1 and β-catenin are known to be involved in Wilms'

Abbreviations: WT, Wilms' tumor; COX-2, Cyclooxygenase-2; *Wt1*, Wilms' Tumor 1 gene; Igf2, Insulin Growth Factor2; HIF-1α, Hypoxia-inducible factor 1-alpha; IDO, Indolamine 2, 3-dioxygenase; TGF-β, Transforming growth factor beta; TNF-α, Tumor necrosis factor alpha; pDCs, Plasmacytoid Dendritic Cells; Tregs, T regulatory Cells; RPPA, Reverse Phase Protein Array

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tumorigenesis [2], the exact molecular pathogenesis of this cancer is unclear. Elucidation of these mechanisms will substantially improve our understanding of the pathways involved in WT tumorigenesis and aid in the development of more effective therapies.

There has been increasing awareness and emphasis on the role of the tumor microenvironment in tumorigenesis and, potentially in cancer therapy. It is now believed that characterizing the components within the tumor microenvironment that are involved in tumor growth and progression and the pathways that regulate them will lead to identification of novel prognostic markers and tumor-associated therapeutic targets. Though the relationship between chronic inflammation and cancer and the components of inflammation that are responsible for tumor development have been reported in various cancers, no information is available on the role of these critical components in WT development. To do this, we previously defined and characterized the inflammatory microenvironment in human WT where we have observed the robust expression of inflammatory marker Cyclooxygenase-2 (COX-2) [3] in almost all the tumors we have analyzed (3). Our purpose in the current study is to extend those studies to analyze the contribution of the microenvironment in particular, the role of COX-2 and its pathway components in WT progression using a mouse model of Wilms' tumor in detail. We thus characterized the tumor microenvironment in a mouse model of WT that was generated in our laboratory by *Wt1* ablation and *Igf2* up regulation [4]. In these mouse tumors and in littermate control kidney tissues, we evaluated and defined the expression of various inflammatory markers by immunohistochemical (IHC) analysis; isolated and identified the inflammatory cells regulated by the cyclooxygenase-2 (COX-2) pathway by flow cytometry; and quantified expression of various inflammatory chemokines, chemokine receptors, and inflammatory cytokines by quantitative polymerase chain reaction (qPCR) and other methods. Our results indicated that the WT tumor microenvironment is enriched with immunosuppressive inflammatory cells, trafficking of which is regulated by COX-2. The important role of these inflammatory cells in creating immunosuppressive tumor microenvironment and the role of COX-2 in immunosuppressive immune cell production and trafficking are also elucidated. This new understanding of the mechanisms underlying WT progression will be useful in planning for the use of specific inhibitors to treat these tumors.

Materials and Methods

Animal Experiments

All animal experiments were approved by the Institutional Animal Care and Use committee of The University of Texas MD Anderson Cancer Center. *Wt1* was inactivated mosaically or almost completely in *Wt1−/flCre-ER* embryos by in utero treatment of pregnant mice with tamoxifen (1 mg/40 g body weight) at E11.5. This treatment resulted in Cre-recombinase activity in approximately 5–10% of cells. All embryos carried a maternally inherited *H19*$^{-}$ allele that results in up regulation of *Igf2* as a result of loss of imprinting and biallelic expression of *Igf2*.

Human Tissue Samples

Human WT tissues and autologous normal kidney specimens were obtained from 16 WT patients aged 7 to 66 months at the time of diagnosis. Eight of the patients were males and eight were females, and one patient had bilateral disease. Of these 16 patients, 4 were at stage. IV, 4 were at stage III, 3 were at stage II, and 5 were at stage I of WT disease. Informed consent was obtained from each patient's parent or guardian. Studies were approved by the Institutional Review Board and in accordance with an assurance filed with and approved by the US Department of Health and Human Services.

Immunohistochemical Analysis of Inflammatory Markers

Tissue processing. Tumor tissues and control kidney samples were collected from mice and fixed in 10% neutral-buffered formalin. After 12–16 hours, formalin was replaced with 70% ethanol; the samples were subjected to dehydration by a series of graded alcohols and xylene and then embedded in paraffin. The tissues were then cut in 5-μm sections using a Leica 2135 microtome.

Analysis. Sections were deparaffinized and used for Hematoxylin and Eosin staining and IHC analysis of various markers. The paraffin-embedded tissue sections from mice and humans were deparaffinized in xylene, rehydrated sequentially in a graded series of ethanol's (100, 90, and 70%), and placed into 1% phosphate-buffered saline solution (PBS; pH 7.4). The epitope retrieval was performed by heating for 45 minutes in 1 mM Tris EDTA pH 9.0 buffer in a water bath at 95–100°C for COX-2 and hypoxia-inducible factor 1-alpha (HIF-1α), in 10 mM Tris+ 0.5 mM EGTA pH.9.0 buffer for phospho-ERK1/2, and in 1 mM EDTA pH 9.0 buffer for p-STAT3. The sections were cooled at room temperature in the buffer for 1 hour, washed three times with 1× PBS for 5 minutes/wash, and incubated with 10% normal serum to block nonspecific protein binding. Expression of inflammatory markers was detected by applying the following antibodies: polyclonal goat anti–mouse/human COX-2, 1:100 dilution (Santa Cruz Biotechnology SC-1747); polyclonal rabbit anti–mouse/human HIF-1α, 1:100 dilution (Novus Biologicals NB100–479); monoclonal rabbit anti–mouse/human phospho-p44/42 ERK1/2, 1:100 dilution (Cell Signaling Technology Cat #4376); and rabbit monoclonal anti–mouse/human phospho-STAT3 (Cell Signaling Technology Cat #9145); sections were incubated in 2.5% appropriate normal serum overnight at 4°C in a humidified chamber. The sections were then washed three times with 1× PBS (10 minutes/wash) and incubated with the appropriate secondary antibody IgG (H + L) solution (biotinylated goat anti-rat IgG 1:500, biotinylated goat anti-rabbit IgG 1:500, biotinylated goat anti-mouse IgG 1:500, or biotinylated rabbit anti-goat IgG 1:500, respectively) in 2.5% normal serum for 1 hour at room temperature. An optimized control-positive tissue section and a negative control section for endogenous staining by omission of the primary antibody were included with every immunostaining batch. The bound positive cells were detected by applying the Vectastain Elite ABC reagents (Vector Laboratories, Inc., Burlingame, CA) and avidin DH: biotinylated horseradish peroxidase H complex with 3, 3-diaminobenzidine (Vector Laboratories, Inc.) and counterstaining with Mayer hematoxylin (Fisher Scientific, Fair Lawn, NJ). Images were captured by an Olympus BX60 microscope.

Flow Cytometry

Single cell preparation. To prepare single-cell suspensions from tumors and control kidneys for flow cytometry analysis, tumors and normal kidneys were cut, washed with RPMI medium to remove blood, and weighed. Tissues were minced and digested with 10 mL of digestion medium (Type IV Collagenase 1 mg/mL in RPMI 1640 medium) by incubating the fragments for 30 minutes [Sigma-Aldrich, St. Louis, MO] in 100-mL conical trypsinization flasks with tumor digestion media. During this incubation, the tissues were subjected to

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