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Inflammation-driven colon neoplasmatogenesis in uPA-deficient mice is associated with an increased expression of Runx transcriptional regulators

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ABSTRACT

Deregulation of the bone morphogenetic protein (BMP) pathway has been documented in colorectal cancer (CRC). Previously, we investigated possible associations between urokinase-type plasminogen activator (uPA) deficiency and expression of extracellular constituents of BMP signaling in a newly developed mouse model of inflammation-driven intestinal neoplasmatogenesis, in which chronic colitis and CRC are induced using dextran sodium sulfate (DSS). In this report, we explored the contribution of intracellular components of Smad-mediated BMP signal transduction using the same model. Interestingly, upon DSS treatment, we noticed an overexpression of *Runx1/2/3* transcription factors in both wild-type and uPA-deficient mice. Moreover, *Runx1* and *Runx2* expression levels exhibited an even higher increase in DSS-treated/uPA-deficient mice as compared to DSS-treated/wild-type animals. In all experimental conditions, in situ investigation of Runx-expressing cell types, revealed detection of all three Runx in the immune cells, yet in the DSS-treated/uPA-deficient mice Runx1 and Runx2 were also identified in the preneoplastic epithelium of advanced high-grade dysplasia and carcinoma in-situ colonic lesions. Finally, the uPA-deficient pro-tumorigenic colitic microenvironment exhibited increased levels of the Runx-induced target genes *Snai2*, *Bim* and *Claudin1*, known to have a role in tumor development and progression. These findings suggest that the absence of uPA correlates with increased levels of Runx transcriptional regulators in a way that promotes inflammation-associated carcinogenesis.

1. Introduction

Urokinase-type plasminogen activator (uPA) is a serine protease mostly known for its key role in fibrinolysis, tissue remodeling and cell migration [11,9]. uPA is involved in the degradation of a wide range of extracellular matrix (ECM) components, a fact that abides by its role in tumor progression and metastasis [10,13,32]. Its proteolytic activity enables cancer cells to invade the extracellular matrix and basement membranes and metastasize, and further enhances tumor growth, stroma remodeling and angiogenesis [10,13,32]. To date, uPA is considered an unfavorable prognostic indicator and a promising therapeutic target for many types of cancer [17,38,39].

uPA catalyzes the conversion of plasminogen to plasmin, which in turn activates downstream proteases and growth factors, such as the transforming growth factor-β (TGF-β) [21,37]. TGF-β is known for its dual role in carcinogenesis, acting as a tumor suppressor in the early stages of tumor development and as a tumor promoter in later, more advanced, stages [1,22]. Based on this interlink between uPA and TGF-β, we recently introduced a novel model of inflammation-driven colorectal cancer (CRC) in order to investigate possible involvement of uPA in the early stages of the disease [27]. Chemical induction of chronic colitis using dextran sodium sulfate (DSS) revealed that uPA deficiency promotes inflammation-associated intestinal neoplasmatogenesis. Compared to their wild-type counterparts, DSS-treated uPA-/- mice had an altered colonic mucosa inflammatory milieu – characterized by more neutrophils and macrophages, less regulatory T cells (Treg), significantly upregulated cytokines (including IL-6, IL-10, IL-17, and TNF-α), and lower levels of active TGF-β1 – and more advanced

Abbreviations: BMP, bone morphogenetic protein; Cldn1, Claudin1; Co-Smad, common-partner Smad; CRC, colorectal cancer; DSS, dextran sodium sulfate; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; GALT, gut-associated lymphoid tissue; Id, inhibitor of differentiation; IHC, immunohistochemistry; I-Smads, inhibitory Smads; MLN, mesenteric lymph nodes; R-Smads, receptor-regulated Smads; Runx, Runt-domain transcription factor; Smurf, Smad-ubiquitination regulatory factor; TGF-β, transforming growth factor-β; uPA, urokinase-type plasminogen activator

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preneoplastic changes that led to the formation of large polypoid adenomas in the colon [27]. These results challenged the current notion that views uPA as only a tumor promoter, as they suggested that it may also act as a tumor suppressor, at least in the early stages of inflammation–associated intestinal carcinogenesis. The initiation of CRC in the above described model was attributed, at least in part, to the presence of augmented inflammatory protumorigenic events, further enhanced by the inadequate tumor suppressive effects of the low amounts of active Tgf-β, due to the lack of uPA [27].

The TGF-β superfamily of cytokines comprises of – other than the TGFs – more than 30 ligands, including bone morphogenetic proteins (BMP) [51]. Originally found as important regulators of skeletal homeostasis during development, BMPs are currently being implicated in tumorigenesis in a bi-directional mode, following the TGF-β paradigm [14,45]. Since BMP pathway deregulation has also been documented in CRC [19,29,30], in a recent study of our group we sought to investigate possible associations between uPA deficiency and the expression of extracellular constituents of BMP signaling, including BMP ligands/receptors and inhibitors, in our model of inflammation-driven intestinal neoplasmatogenesis [26]. In the DSS-treated wild-type mice, the preneoplastic lesions which did not eventually evolve to adenomas, were found to reside in an inflammatory microenvironment characterized by a balanced upregulation of both BMP ligands i.e. *Bmp4/7* and BMP inhibitors, such as *chordin*, *noggin* and *gremlin-1*. In the uPA-deficient tumor-promoting inflammatory microenvironment, however, there was a clear evidence for BMP pathway suppression as identified by the combined reduction of *Bmp4* and an even higher increase of *gremlin-1* expression. These findings proposed that BMP pathway suppression in CRC may be associated with very early stages of the preneoplasia-to-neoplasia sequence of events [26].

Continuing our effort to unravel the uPA/BMP inter-relationship, in the present study we explore the contribution of the intracellular relay of BMP signaling in our uPA-deficient mouse model of inflammation-associated CRC. BMPs signal through transmembrane receptors and activate both Smad- and non-Smad-dependent signal transduction [15,40]. Here, we focus on Smad-dependent signal transduction and investigate gene expression of multiple participants of this BMP-triggered intracellular pathway, from R-*Smads* down to target genes.

2. Materials and methods

2.1. Animals and tissues

Total RNA from colon tissue samples and paraffin-embedded colon used in the present study was retrieved from earlier experiments [27]. Mouse housing, experimental design, and tissue collection/ handling have been described in detail elsewhere [27]. Briefly, for the induction of chronic colitis, 3.5% DSS was given in the drinking water of 8- to 10-week-old BALB/c mice for 1 week followed by 1 week of regular water. This cycle was repeated three times. Wild-type and uPA-deficient mice were either treated or not with DSS. Mice were sacrificed 1 week after DSS treatment. Experimental groups and numbers of mice per group were as follows: wild-type, n = 5; uPA-deficient, n = 5; wild-type/DSS, n = 6; uPA-deficient/DSS, n = 7.

2.2. Quantitative gene expression analysis

Real-time PCR based on the SYBR Green chemistry was used to quantitatively determine gene expression, as previously described [27]. Primer sequences, their positions within the corresponding genes, and amplicon sizes for 20 members of the intracellular BMP signaling pathway, as well as the ones for the housekeeping gene Gapdh, are presented in Table 1.

2.3. Immunohistochemistry and morphometry

Formalin-fixed, paraffin-embedded colon tissues were cut at 5 μm and stained with immunohistochemistry (IHC). Primary antibodies for IHC included rabbit polyclonal antibodies against Runx1 (Abcam, Cambridge, UK), Claudin 1 (Abcam) and Runx3 (Origene Technologies Inc., Rockville, MD, USA) or rabbit monoclonal antibody against Runx2 (Abcam). Heat-induced antigen retrieval was performed with citrate buffer, pH 6, for Claudin1, and with EDTA buffer, pH 8 for Runx1, Runx2 and Runx3. Rabbit primary antibody binding was detected with goat anti-rabbit polymer HRP (ZytoChem Plus, Berlin, Germany). Color was developed with DAB substrate-chromogen system (Biogenex, Fremont, CA, USA) and tissues were counterstained with hematoxylin.

For quantitative histomorphometry, IHC-positive immune cells (Runx1, Runx2 and Runx3) or pixels (Claudin1) were counted in images of ×40 representative high power fields using the ImageJ image processing and analysis program (NIH, Bethesda, MD), as previously described [27].

2.4. Statistical analysis

Relative gene expression and histomorphometry data were compared between groups using the non-parametric Mann-Whitney *U*-test and GraphPad Prism version 6.01 for windows (GraphPad Software, San Diego, CA). Data were presented as bar graphs depicting the mean and standard error of the parameter assessed for each experimental group.

3. Results

3.1. Expression levels of receptor-regulated Smads and their modulators are not affected by uPA deficiency and/or DSS treatment

First, we investigated gene expression levels of the receptor-regulated Smads (R-Smads) known to be activated after BMP ligand binding, i.e. *Smad1/5/8*. Since no prominent changes were observed in any of the three molecules following DSS treatment in either wild-type or uPA-deficient mice (Fig. S1A–C), we next sought to examine whether uPA deficiency and/or DSS treatment affect the dynamics of intracellular Smad regulatory elements. Smad-mediated signaling can be negatively regulated by both inhibitory Smads (I-Smads), namely Smad6 and Smad7, and by Smad-ubiquitination regulatory factors 1 and 2 (Smurf1 and Smurf2) [15,40]. I-Smads compete with R-Smads for binding to activated type I receptors and form non-functional complexes with Smad4. Smurfs, on the other hand, inhibit BMP signaling by interacting with and degrading R-Smads and activated type I receptors [15,40]. In our mouse model of inflammation-associated CRC, both *I-Smads* and *Smurfs* showed comparable levels of expression in all experimental groups (Fig. S2A–D), suggesting that Smad modulation does not comprise an intracellular BMP regulatory mechanism in this model.

3.2. uPA deficiency enhances DSS-mediated upregulation of Runx1/2 transcription factors

Next we explored gene expression patterns of molecules that interact with R-Smads after their translocation to the nucleus, such as transcription factors and transcription co-activators/co-repressors. We first investigated the expression of the transcription co-activators *p300* and *CBP*, and the transcription co-repressors *Ski* and *SnoN*, all known to participate in BMP intracellular signaling by interacting with R-Smads within the nucleus and regulating transcription of target genes [15,40]. Our results revealed that neither DSS treatment nor uPA deficiency affected the expression of any of these transcriptional regulators (Fig. S3A–D).

The Runt-domain transcription factors, Runx1, Runx2 and Runx3, seemed to be alternative candidates, as they are also known to interact

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